PAPER-BASED EXTRACTION AND AMPLIFICATION OF BACTERIAL GENOMIC DNA TO MONITOR FOOD SAFETY

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree of Master of Applied Science

McMaster University MASTER OF APPLIED SCIENCE (2020) Hamilton, Ontario (Chemical Engineering)

TITLE: Paper-based extraction and amplification of bacterial genomic DNA to monitor food safety

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NUMBER of PAGES: xi, 72

Abstract

The development of rapid detection methods for bacterial contamination in the industrial agriculture sector is vital for improving food safety and public health. Furthermore, it is important to tailor these rapid methods for low-resource settings, because the majority of foodborne illness outbreaks occur in developing nations. Currently, the most widely used methods rely on nucleic acid testing using the polymerase chain reaction (PCR). This detection reaction provides repeatable results, is highly sensitive, and is highly specific, as it can detect a single strain within a species. However, PCR is reliant on proper sample pre-treatment to remove inhibitory contaminants which can affect downstream results, which leads to a trade-off between detection time and sensitivity of results. Rolling circle amplification (RCA) is another potential detection reaction which has the same advantages and is also better suited to low-resource settings, as it works at room temperature.

This thesis reports on the development of a rapid sample preparation method that can be seamlessly integrated into simple PCR and is also well suited to low-resource settings due to the low cost and high availability of the required reagents. A modification of the hot sodium hydroxide plus tris (HotSHOT) lysis reaction was implemented to extract genomic DNA (gDNA), which was then captured onto cellulose filter paper, allowing for multiple samples to be simultaneously processed in under 30 minutes. This pre-treatment can even recover gDNA for detection from samples that would have caused complete inhibition of PCR. The calculated limit of detection (LoD) of extraction followed by simple PCR was similar to that of government-approved commercial kits, without needing a lengthy bacterial enrichment step. Improvements are needed to make this a truly quantitative detection system. Finally, our paper-based pre-treatment was integrated into an RCA reaction to detect at least 10^5 cells, which provides proof-of-concept for combining paper-based sample preparation with isothermal amplification of target nucleic acids.

Acknowledgements

First and most importantly, I would like to thank my supervisor Dr. Carlos Filipe, for his unending guidance and support, not only over the course of my M.A.Sc. research, but throughout my journey at McMaster. Thanks for helping me with a solution or an explanation for every problem I had, this thesis would not have been possible without you. You always had faith in me, even at times when I did not, and words cannot express how important that was. Thanks for being the best first boss I could ever ask for; I am, and forever will be, grateful for your support.

I am also incredibly grateful to Dr. Monsur Ali, a well renowned postdoctoral fellow who taught me almost everything I know about good laboratory technique. Your guidance helped me break through several roadblocks when my results were not following the anticipated theory. Thanks for always greeting my questions with a smile, and for all your intelligent suggestions.

I am very appreciative of the hard work of my undergraduate research assistant, Kate Hardman, who helped gather and process data. Your persistently positive attitude was a refreshing presence and it was great to see you learn techniques rapidly, I see a bright research career ahead! Thanks for always having a smile, whether it was a long day in the lab or a fun Filipe fro-yo Friday!

I must also acknowledge several other graduate students, Mitchell Ross, Nicole Amaral and Taylor Goostrey, for their constant support and always being there to bounce ideas off one another.

A special thanks goes out to my good friend Sophia Emerson, who was there throughout my whole McMaster journey, minus the first and last year. Diamonds are made under pressure, and that's how we made it through. Thanks for being a great study buddy, project partner, motivator and consult, I could not have done it without you!

I owe a shout out to my close friends Joseph Alford, Joshua Bone, Leviticus Brown, Ian Gough, and Cornelis van Steekelenburg, for sticking with me through thick and thin and helping me overcome any rough situations I have come across. It took a while, but we all made it out!

I am endlessly grateful to my family for the support they have given me and the lessons they have taught me. My mum, Neomi, has been a prime example for how unbreakable the human spirit is and how it is possible to carry on even in the toughest of times. My father, Alfredo, has taught me a lot about recovering from failures to reach an eventual success. Both of them also imparted a priceless amount of wisdom, morals, and belief in me, without which I would not be where I am today. My oldest sister, Dr. Annalise D'Souza, has been an exemplary role model of academic achievement and I hope to some day embody her brilliance and persistence. Your hard-working spirit is unmatchable and was truly inspiring to me. Last, but not least, my sister, Alexia, who's youthfulness was always a welcome reminder to step back and not take things too seriously. Thanks for backing me and listening to me explain my work, hopefully your little brother has made you proud.

Table of Contents

Table of Figures

Table of Tables

List of Acronyms & Abbreviations

BSA: Bovine serum albumin

CDC: Centre for Disease Control

CFIA: Canadian Food Inspection Agency

cfu: Colony forming unit

DALY: Disability Adjusted Life Years lost

dNTPs: Deoxy-ribonucleotide triphosphates

DOE: Design of experiments

EAEC: Enteroaggregative *E. coli*

EC K12: *Escherichia coli.* strain K12

EDTA: Ethylenediaminetetraacetic acid

EFSA: European Food Safety Authority

ELISA: Enzyme-linked immunosorbent assay

EPEC: Enteropathogenic *E. coli*

ETEC: Enterotoxigenic *E. coli*

gDNA: Genomic DNA

HotSHOT: Hot sodium hydroxide and tris

LAMP: Loop-Mediated Isothermal Amplification

LFA: Lateral flow immunoassay

LoD: Limit of Detection

NASBA: Nucleic Acid Sequence-Based Amplification

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

- mPCR: Multiplex PCR
- qPCR: Quantitative/Real-Time PCR

PEG: Polyethylene glycol

PHAC: Public Health Agency of Canada

PoC:	Point of Care (as in diagnostics)
RCA:	Rolling circle amplification
SN:	*Salmonella enterica* serovar Newport
STEC:	Shiga-toxin producing *E. coli*
TSB:	Tryptic soy broth
WHO:	World Health Organization

Declaration of Academic Achievement

The majority of the experimental work presented in this thesis was developed, executed, analyzed, and written by the author in consultation with Dr. Carlos Filipe, except for the following:

Chapter 2: Some gel electrophoresis experiments were executed and imaged with the help of my undergraduate research assistant, Kate Hardman.

Chapter 1: Motivation and Research Objectives

1.1 Epidemiology & Statistics on Global Food-borne Illness

In 2015, the World Health Organization (WHO) conducted the largest ever study on global food-borne illness and estimated that roughly 1 in 10, or 600 million people, suffer from foodborne illnesses every year resulting in roughly 420,000 deaths and 33 million healthy years of life lost (DALY) annually [1]. The Public Health Agency of Canada (PHAC) estimates that somewhere between 11-13 million Canadians annually suffer from foodborne bacterial diseases[2]. The disease burden of food-borne illness disproportionately affects developing countries**,** but still maintains a significant presence in developed nations due to the globalization of the industrial food system, which provides favourable conditions for the international spread of food-borne pathogens[1].

Although the reported numbers of food-borne disease outbreaks have decreased in recent years, there is evidence that this could be due to a higher proportion of outbreaks going unsolved. As an example, between the years 2002-2011, the majority of food-borne illness outbreaks (57-68% of annual outbreaks) investigated by the Centre for Disease Control (CDC) went unsolved[3]. The definition of an outbreak in this context is when more than one person becomes ill from the same source[4]. Additionally, despite substantial improvements being made to public health in recent decades, this progress has not been consistent between developed and developing countries[5]. Roughly half of the global population does not have basic sanitation, about a third do not have consistent access to electricity, and roughly 1 in every 6 people does not have access to clean drinking water and basic healthcare[5].

The majority of the food-borne illness impact falls on poorer regions such as Africa and South-East Asia, which is particularly concerning since many countries in these regions do not

have adequate measures in place to properly study the epidemiology of food-borne illnesses[1]. Another important concern is that many foodborne illness incidents happen intermittently and only affect a few individuals in large populations, and these cannot be accounted for by routine epidemiological surveillance or outbreak investigations[6]. Thus, the total number of cases worldwide is often vastly underestimated, with even the most visible outbreaks in poorer regions slipping under the radar and often only getting detected if they have major impacts on public health or their nation's economy[1].

It is estimated that almost every person will develop a foodborne or waterborne illness at least once, if not multiple times, over the course of their lifetime[7]. One especially distressing statistic to highlight the severity of the current state of the foodborne illness crisis is that 43% of the disease burden is borne by children under the age of 5, and this higher proportion is common across both developing and developed nations[1]. Another age demographic that is particularly susceptible to these diseases is the elderly, although this isn't very well accounted for in the DALY metric since they have already lived out most of their lives. It is also important to note that the DALY metric underestimates the total effect of this situation, not only because cases are underreported, but also since it measures the lost years of life relative to national averages for life expectancy, and life expectancy in developing nations would significantly be increased if better systems for food safety and quality control were introduced in these regions[1].

1.2 Pathogenic Causes of Bacterial Food-borne Illness

With each decade since the advent of foodborne disease surveillance, the list of responsible pathogens has grown longer[8]. However, it is estimated that out of the 420,000 total annual foodborne disease deaths, 124,000 can be attributed to *Salmonella* spp., and a further 63,000 can be attributed to various strains of *Escherichia coli*, making these two the deadliest foodborne

bacteria[1]. It should therefore come as no surprise that they are also the most prominent foodborne pathogens worldwide[9]. It is worth noting that the majority of deaths attributed to both pathogens occurred in the previously mentioned regions of Africa and South-East Asia, which significantly contributed to these regions bearing the largest proportion of the burden of global foodborne illness[1]. However, these two pathogens also caused a large proportion of foodborne disease in developed nations too, especially *Salmonella*, which was prevalent in every region worldwide[1]. The ability of pathogens to persist in the environment is a crucial determining factor in the risk they pose for human infection[10], and both of these bacteria exhibit incredible persistence in a variety of conditions[9]. These two types of bacteria are also the most heavily regulated and monitored[9] and were thus selected for further analysis.

1.2.1 E. coli

E. coli are incredibly common gut bacteria that colonise a wide range of host species, including all major livestock, wild animals and even humans[9]. These colonisations are often commensal in the host, and are in fact often necessary for a healthy gut microbiome[11]. There are six main categories of *E. coli* strains that cause disease, with the most prominent being Shiga-toxin producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and enteroaggregative *E. coli* (EAEC)[9].

It is when these pathogenic strains of *E. coli* are passed from one organism to another, most often through contact or contamination with faeces, that diseases can start to arise[1]. Strains that are commensal to one type of host may be deadly to another, and thus many species that are pathogenic to humans develop asymptomatically in other animals[9]. As an example, epidemiological studies have frequently discovered STEC living in the intestinal tract of healthy livestock cattle which act as a reservoir for these bacteria[12]. Among the STEC strains, those

containing O groups, especially *E. coli* O157, have become an important source of concern for human food-borne intestinal disease[9]. In fact, one of the most frequently occurring causes of water-related outbreaks is *E. coli* O157:H7[1]. However, this strain had not been recorded before 1982[9], and it appears new STEC strains of this species are emerging, since the rates of STEC disease caused by strains other than O157 have increased annually for the last decade[13]. This is likely due to the innate ability of *E. coli* to adapt to changing environments and stresses and continually evolve new varieties that have never been previously discovered or characterized, allowing for colonization of unrecognized niches[9].

1.2.2 Salmonella

Salmonella spp. are also intestinal tract bacteria that commensally colonise a variety of host species, especially birds, humans and all the major livestock species[9]. While the case rates per capita of most other bacterial foodborne diseases in the US have gone down in the last 15 years, the rates for *Salmonella* have essentially remained constant, thus maintaining its status as the most prevalent and problematic food-borne bacterial pathogen[13]. Infection with *Salmonella* (often called salmonellosis) can cause a variety of diseases ranging from mild gastroenteritis to Typhoid fever, which can lead to severe complications and possibly death[14]. Milder cases are often misdiagnosed or unreported, as they can be easily confused with other causes of gastroenteritis[14]. The CDC estimates that salmonellosis causes roughly 1.2 million illnesses and 420 deaths annually in the US alone[6].

Salmonella enterica serovar Newport, which is the serotype I used in my experiments, is responsible for the third highest number of salmonellosis infections of patients in the United States[13]. Salmonellosis is especially problematic in children under 5, who do not yet have fully developed immune systems and are the most likely age group to get infected by far[15], especially

for infants that are not breastfed[9]. *Salmonella* infection incidence rates in the US are almost 3 times as high as the next most prevalent foodborne bacteria (*Campylobacter* spp.) and more than 10 times higher than most other foodborne bacteria for this particularly vulnerable age group[13]. What makes this problem even more challenging to tackle is the fact that *Salmonella* spp. are remarkably adaptable organisms that perpetually evolve to develop tolerance to environmental stresses, allowing for colonization of unrecognized niches[9]. This ability to evolve new resilient subspecies, when added to the widespread nature of known *Salmonella* spp., makes controlling and detecting these bacteria an important step towards achieving food safety[15].

1.2.3 Antibiotic Resistance

An issue that is becoming increasingly prevalent in bacterial pathogens in the food chain is antibiotic resistance[9]. The widespread use of antibiotics in industrial agriculture and human healthcare has led to rising rates of emergence of antibiotic resistant bacteria and antibiotic resistance bacterial genes[16]. It has been shown that antibiotic use in livestock commonly exerts selection pressure for multiple resistance genes in *Salmonella* serotypes such as Typhimurium, Virchow, Derby and Newport[9]. Over half of all antibiotics used in the US are given to livestock, and up to 95% of each dose administered to humans and animals has been shown to be excreted in an unaltered state, which can lead to bioaccumulation of these drugs in the environment[16]. This can be problematic in food processing systems that rely on these antibiotics as a major control measure to kill pathogenic bacteria, since resistant bacteria can bypass this treatment step and still cause foodborne illnesses downstream, and severe cases would then become difficult to treat due to the antibiotic resistance trait.

Rising rates of resistance are especially problematic since antibiotic use in one ecological compartment can have effects on the resistance status elsewhere[17]. Thus, an increasing rate of

emergence of antibiotic resistant bacteria and resistance genes in the agricultural sector poses a potentially severe threat to both public health and the fate of environmental ecosystems[7]. Various studies have also shown that antibiotic resistance genes can be horizontally transferred through plasmid exchange between different strains of the same bacteria, and also between completely different species[17-20]. This plasmid transfer can occur in the intestinal tracts of humans & livestock, but also in environmental matrices such as water & soils, and even on surfaces that come into contact with food such as kitchen towels & cutting boards[17-20]. It has also been shown that commensal *E. coli* of healthy humans serve as a reservoir of resistance genes, and one study of a few similar individuals (n=20) who exhibited different combinations of only 4 resistant strains of *E. coli*, discovered substantial diversity in resistance phenotypes, resistance genes and gene combinations[11]. When resistant bacteria are released into the environment via faeces, they can evolve and transfer plasmids to propagate resistance further, and this resistance spread is incredibly difficult to track[17,18]. Thus, it is incredibly important to limit the amount of antibiotic resistant bacteria that emerge through industrial agricultural systems to prevent the downstream spread of resistance plasmids and development of new resistant strains of bacteria[17].

1.3 Vectors of Transmission of Food-borne Illness

1.3.1 Globalization Presents New Challenges

Food that is mass-produced through industrial agriculture is transported through a large, complex distribution network. This far-reaching network can vastly magnify the effects of even the most localized contamination events, since there are a lot of stages where cross-contamination can occur[8]. The truly globalized nature of the industrial food production system has introduced many new potential sources of contamination, which has made it increasingly challenging to conduct thorough quality control checks[1]. Since contamination can occur at any point along the

food supply chain, it is not sufficient to only test for contamination at the source but rather have some form of regulatory test at each stage from farm to fork, especially just before the food reaches the consumer[10]. Although it may seem like a sensible extension of this reasoning to only test food products right before they reach the consumer (which would be less resource intensive), it is best to test and remove contaminated food as early as possible so as to reduce the risks associated cross-contaminating other similar items, or worse, causing cross-contamination of other types of items at a processing facility. Additionally, having rapid tests at many stages provides an overview of the whole supply chain and allows for more precise tracking of products, which is critical to pinpoint potential sources when an outbreak occurs[21]. Figure 1.1 below highlights some potential sources of contamination along with their routes of transmission to humans. These will also be expanded upon further in this section.

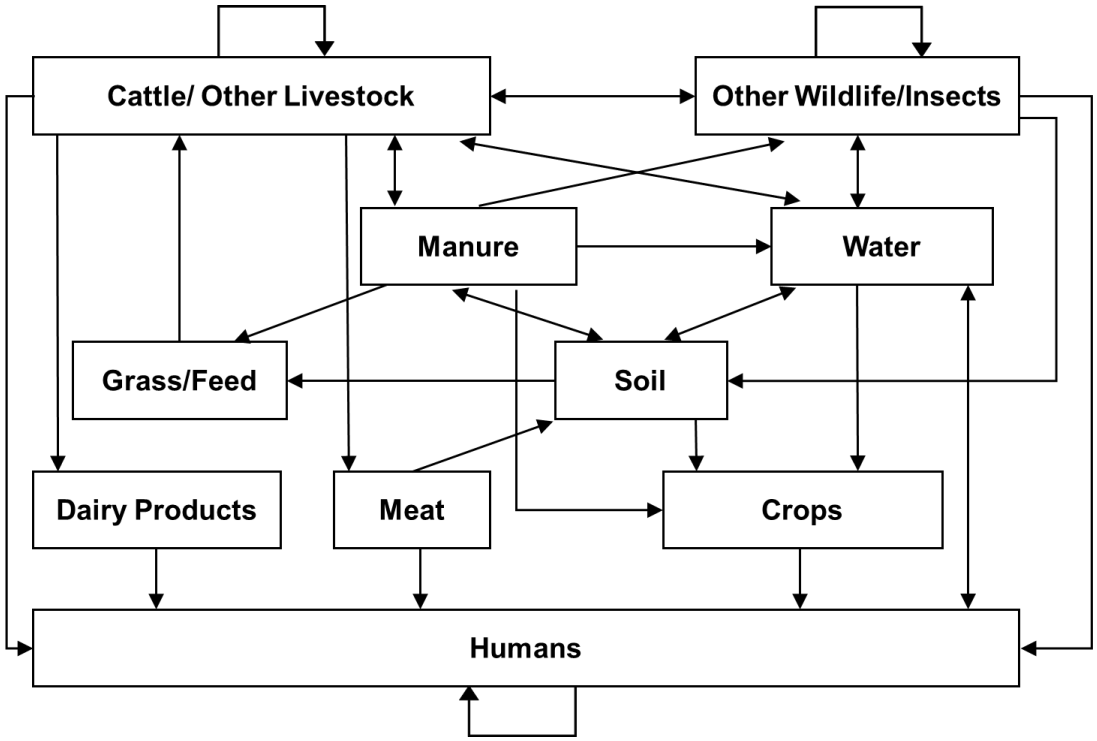


Figure 1.1 A schematic representation of the complex network of transmission routes of foodborne pathogens. It is important to note that these pathogens can be transmitted to humans through other methods of exposure besides food, but they often come from the same microbial reservoirs. The interconnectedness highlights the ease of pathogen spreading. Adapted from [24] and edited.

1.3.2 Environmental Contamination (Water, Soil, Manure & Animal Feed)

The first potential sources of contamination occur at the farm, with contaminated raw materials leading to contaminated products. The precise nature of these environmental risks varies from one farm to the next, as they are dependent on factors such as climate, land-usage interactions, and raw material sources[10]. This is especially important in the case of farms for produce, where pre-harvest contamination sources must be controlled, since it is challenging to treat produce once it has been contaminated[10].

The first important pre-harvest hazard is the water used to irrigate crops or hydrate animals. The microbial safety of this water is dependent on the source, with collected rain and deep groundwater being the safest[22]; followed by shallow groundwater, wells, and surface groundwater, which can be contaminated by surface runoff[10]; and finally, raw or inadequately treated wastewater, which despite potentially containing nutrients for growth will often also contain high concentrations of microbes[22].

Another important pre-harvest hazard is the soil and manure used for produce cultivation. Most foodborne pathogens favourably survive in cool, moist environments such as soil[23]. Clay soils exhibit enhanced adsorption of microbes, and reduce their die-off rates by forming a barrier to protect against microbial predators and parasites[23]. The rise of industrial agriculture has led to widespread usage of organic wastes to enhance the nutrient content of soils, such as livestock manure, abattoir wastes and municipal and industrial sewage sludge residues[22]. There is also evidence that enteric pathogens, specifically *S. enterica* and *E. coli*, survive longer in mixtures of soil and manure than in the actual manure itself[24]. The primary sources for pathogen entry into soil include animal grazing, using contaminated irrigation water, and organic wastes[23]. Manure

piles may also lead to contaminated run-off being deposited into surrounding fields and waterways[22].

The final important raw material in food and drink production that could potentially cause contamination is animal feed. Feed that has been produced using microbially contaminated ingredients can lead to higher concentrations of these microbes in livestock intestines[25]**.** Since the toxicity of enteric pathogens is host-specific, livestock eating contaminated feed may not present with any symptoms, especially since they are also routinely given antibiotics[25]. This presents another challenge that these animals may harbour antibiotic-resistant bacteria while being asymptomatic and then pass these bacteria on in their manure[25]**.** Manure composition is also largely determined by the formulation of animal feed, as is the microbial content of the manure, and livestock that eat industrial feeds, rather than their natural, fibre-rich plant diets, tend to excrete more *E. coli* O157:H7 and *Salmonella* [24].

1.3.3 Produce Contamination

Although the public opinion is that foodborne illnesses mostly originate from poultry and meat, there has been an increased prevalence in the number of foodborne disease outbreaks associated with fresh produce in recent decades[10], and this category now causes the most outbreaks in the developed world[3]. Additionally, fresh produce was shown to cause the most illnesses per outbreak on average[3]. The bacteria causing the most foodborne illnesses from produce are *E. coli* (especially STEC) and *Salmonella* spp[10]. Even though *Salmonella* are typically associated with poultry and meat, many crops are now produced in developing countries where manure is often used as a fertilizer[24]. In fact, ongoing studies suggest that some *Salmonella* strains have evolved to attach and colonize vegetables despite typically being intestinal pathogens in animals[24,26].

Microbiological contamination of fresh fruits and vegetables is especially important since they are mostly eaten raw or after minimal processing[27]. Contamination may occur through many routes, such as uptake through roots, direct contact of edible surfaces with contaminated water or soil mixtures, splashing of contaminated soil mixtures onto produce during watering, or insects that land on edible surfaces after landing on contaminated soil/manure[22,24].

1.3.4 Meat Contamination

The major bacterial contaminants associated with meat are *Salmonella* and pathogenic *E.coli* for cattle[28] and *Salmonella* for poultry[29], which can live in the intestines of healthy livestock animals[9]. The secretion of these pathogens in animals faeces is increased by a number of stresses, such as noise pollution, high density living conditions, abnormal temperatures, and abnormal diets, and this can allow these pathogens to spread rapidly on farms and in lairage facilities[28]. Animal muscles are the major parts used for human consumption, and although they are sterile in living animals, they can be directly contaminated during the slaughtering process by exposure to skin, intestines, organs, etc.[29] However, indirect contamination through contact with process water or equipment surfaces is also common in slaughterhouses and processing facilities[18,28]. Thus, more processed items, such as minced meats, present a higher contamination risk[29]. As such, diligent processes for slaughtering and carcass handling are essential to prevent the spread of foodborne pathogens, and regular testing of equipment surfaces and final products will allow for quick detection of any possible issues[29].

1.3.5 Processed & Ready-to-eat Foods

Increasing consumer demand for convenience has driven a sharp increase in the production of ready-to-eat foods, especially those that are partially cooked or precooked and chilled. Consumers do not associate these products with being uncooked, and thus are unaware of

pathogenic risks they may pose, so a failure to properly kill bacteria in the production and processing of these types of foods can have drastic consequences[8]. Inadequate procedures for food safety, or a lack of properly following procedures in one of these facilities, can lead to a cross-contamination event with widespread consequences[18], for example. the contamination of precooked airline meals at one facility can cause an outbreak affecting people across the globe[9]. It is thus essential to test these products for safety before they are released to consumers[8].

1.3.6 Contamination from Food Handling & Human Sources

One of the most important sources of foodborne illness is improper handling by humans during food preparation, whether that is at a restaurant, or by consumers[29,30]. Restaurants play a much larger role in contributing to foodborne disease outbreaks, since a single contamination event can affect many customers[8], but it is important to note that foodborne diseases from private homes often go unreported since they are subject to less scrutiny[4]. Recent decades have seen significant growth in the restaurant industry, especially fast food, which has led to an increase in hiring low-wage workers with less training in proper sanitation, along with a wider customer base and thus wider reach of an outbreak[8]. Bad food handling practices can lead to contamination of food preparation surfaces and equipment[17,28-30]. A study has determined that knowledge of safe food handling practices does not always transfer to people's food preparation technique[30].

Arguably the most important vector of human-human transmission of foodborne diseases is through contact with traces of faeces from an infected person[8]. This can be further complicated if there is faecal contamination of drinking water[1], food processing plants[28,29], or restaurants[8], for example, a food service worker handles food without proper hygiene[4,8,30].

1.4 Methods of Detecting Bacterial Contamination

1.4.1 Bacterial Detection is an Evolving Field

Until the most recent decade, the industrial standard tests for detecting bacterial contamination in the food and drink sector all followed the conventional methodology of culturing bacterial cells for detection[31]. This typically involves growing bacteria in a nutrient-rich media solution or on a gel media plate. Food samples are typically homogenized, with an optional step for culture enrichment if the original pathogen concentration is low, following which the mixture is grown on a selective medium that promotes the growth of the pathogen being tested for. Further subtyping studies may also be conducted to narrow down between different strains of the same species[32]. There have been many improvements made to culturing techniques to improve their sensitivity and bacterial recovery, most notably selective agar overlays, wherein bacteria are grown on a nonselective media first before a selective media is applied directly over that[33]. Another major innovation in culturing methods was the invention of 3M™ Petrifilm™ plates which are thin, dry gel films that are easier to store than agar plates, and can be prepurchased instead of preparing the agar in-house[34]. Petrifilm™ plates also offer good repeatability when compared to agar plates[34]. These conventional methods gained popularity as they are generally inexpensive and easily repeatable, however, they are also quite lengthy with each enrichment/growth step requiring at least 8-24 hours[33]. Reducing this detection time is crucial to identify causes of outbreaks and control their spread[31]. Another drawback with these methods is the potential for false negatives due to "the great plate count anomaly", that bacteria which can be cultivated *in vitro* represent only a small proportion of the biodiversity of microorganisms within our natural environment[35].

Although culture-based methods are still the most widely used, there has been continuous development of culture-independent detection methods[36]. These "rapid" methods can be generally classified as biosensor-based, spectroscopic, immunological-based, and nucleic-acid-based, however, it is worth noting that methods within the same subcategory may differ significantly[31]. Figure 1.2 below presents a schematic that compares the steps required for culture-based versus culture-independent bacterial detection methods. When discussing rapid detection methods, it is important to consider the geographical locations where they are being used. Many innovative rapid techniques have been designed for use in well-funded laboratories with refrigerated reagent storage, a constant power supply, a large inventory of reagents and specialized equipment, and a plethora of well-trained personnel[5]. Since the vast majority of the global foodborne disease burden falls on the developing world, it is important to design rapid tests that are cost-effective and simple to perform with basic reagents for optimal detection in low-resource settings[5].

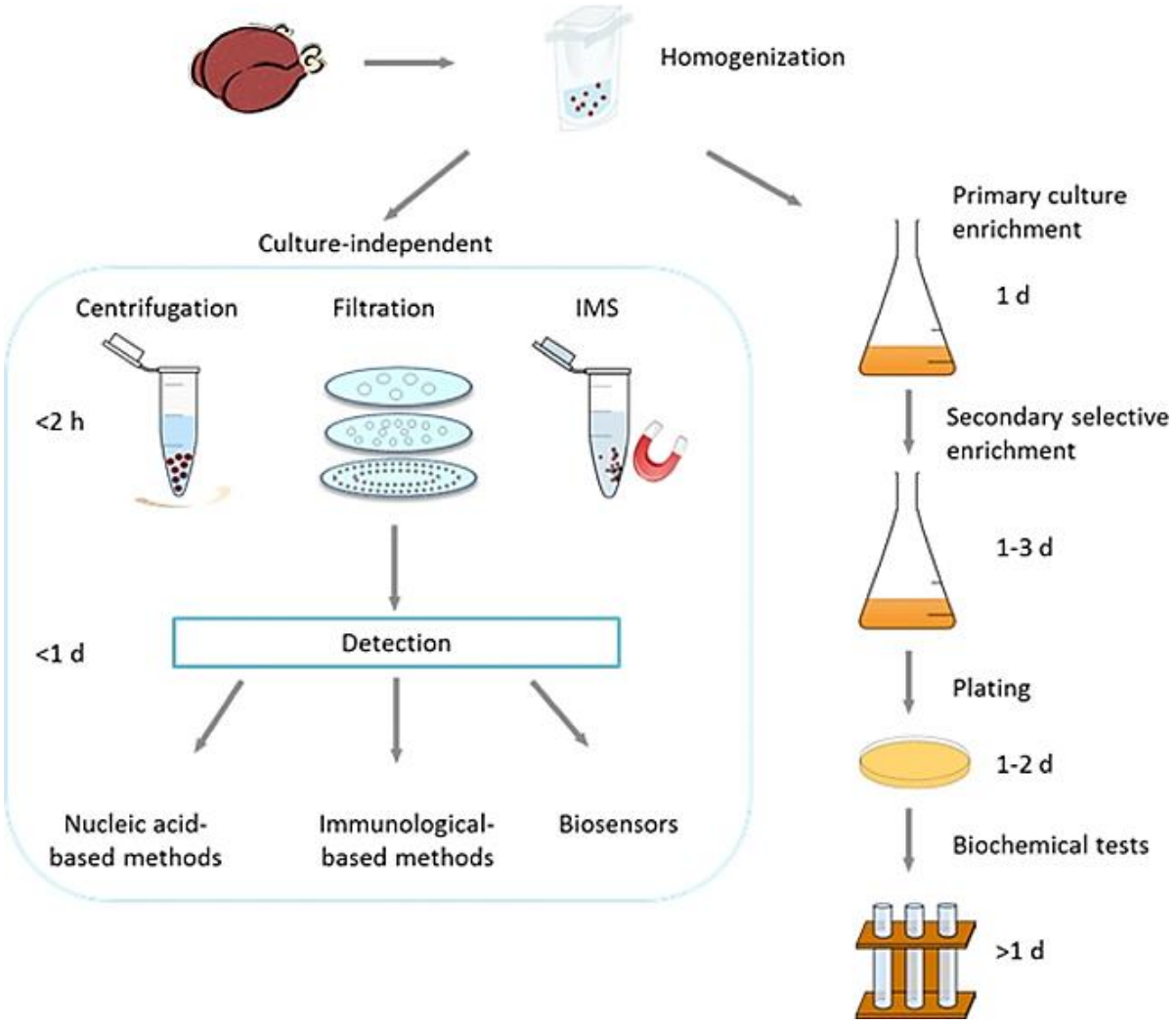


Figure 1.2 This illustration presents a comparison of the steps required for conventional (culture based) bacterial detection methods versus some modern culture-independent methods. Note that the times presented are a rough generalization, as are the schematic images of the steps required. Adapted from [31].

A summary of the general characteristics of rapid detection methods is presented in Table 1.1 below. This highlights the advantages of each method as well as limitations that need to be addressed. This information is also expanded on in the following sections covering each category of detection methods, along with a description as to how they work.

Table 1.1 A summary of currently available rapid methods of bacterial detection, highlighting the advantages and limitations of each. Adapted from [37] and edited.

Category	Detection Method	Advantages	Limitations
Nucleic Acid Testing	**Simple PCR**	• High sensitivity • High specificity • Reliable Results • Automated	• Affected by PCR inhibitors • Difficult to distinguish viable cells
	Multiplex PCR	• High sensitivity • High specificity • Reliable Results • Automated Detection of multiple pathogens	• Affected by PCR inhibitors • Requires DNA purification • Difficult to distinguish viable cells • Primer design crucial
	Real-time PCR	• High sensitivity • High specificity • Rapid cycling • Reproducible • Does not require post-processing	• High cost • Affected by PCR inhibitors • Difficult to distinguish viable cells • Requires trained personnel • Cross contamination
	NASBA	• Sensitive • Specific • Low cost • Does not require thermal cycler • Able to detect viable microorganisms	• Requires viable microorganisms • Difficulties in handling RNA
	LAMP	• High sensitivity • High specificity • Low cost • Easy to operate • Does not require thermal cycler.	• Primer design is complicated • Insufficient to detect unknown or unsequenced targets
	RCA	• High sensitivity • High specificity • Low cost • Easy to operate • Does not require thermal cycler. • Can be conducted at room temperatures	• Requires linking a circle to gDNA

Biosensing Device Mechanisms	**Optical**	• High sensitivity • Enables real-time or near real-time detection • Label-free detection system	• High cost
	Electrochemical	• Automated Can handle large numbers of samples • Label-free detection system	• Low specificity • Analysis may be interfered by food matrices • Many washing steps • Not suitable for analyzing samples with a low number of microorganisms
	Mass-Based	• Cost effective • Easy to operate • Label-free detection • Real-time detection	• Low sensitivity • Low specificity • Long incubation time • Many washing steps • Regeneration of crystal surface may be problematic
Immunological Testing	**ELISA**	• Specific • Can be automated • Can handle large numbers of samples • Allows the detection of bacterial toxins	• Lower sensitivity than culture-based detection techniques • Can give false negatives • May result in cross-reactivity with related antigens • Requires pre-enrichment to produce cell surface antigens • Requires trained personnel • Requires labelling of antigens/antibodies
	LFA	• Low cost • Reliable • Easy to operate' • Sensitive • Specific • Allow the detection of bacterial toxins	• Requires labelling of antibodies or antigens • More qualitative than quantitative

1.4.2 Biosensor-Based Methods

A biosensor is defined as a chemical sensing device made up of a biological or biomimetic sensing component, and a physicochemical signal transducer[32]. A wide array of devices fall under this broad description with many different signal transduction methods available, such as optical, mass-based, piezoelectric, thermometric, and electrochemical[36]. Biosensors often have shorter operating times than other rapid methods, and can also combine immunological or nucleic-acid detection events onto a portable sensing platform[37]. However, this requires the biochemical detection mechanism to be well developed, to avoid propagation of error through the signal transducer[36]. Optical biosensors offer the most precise detection, as well as the ability to detect bacteria in real-time[38]. However, they also present the highest cost and often require the use of expensive spectroscopic machinery[37], thus making them inappropriate for low-resource laboratories. Although mass-based and electrochemical biosensors offer rapid detection at a low cost, and can handle large numbers of samples, their limits of detection of bacterial cells are vastly inferior to other rapid methods[37]. Thus, although biosensors offer vast potential as bacterial detection platforms of the future, they are currently unfit to meet the vast needs of the developing world in this area[36].

1.4.3 Immunological Testing Methods

Immunological detection, as the name suggests, is dependent on antibody-antigen interactions as a means to detect bacteria, with either bacterial cells or their metabolic products serving as antigens for specially designed antibodies[37]. The most commonly reported and heavily developed immunological methods for rapid detection are enzyme-linked immunosorbent assays (ELISA) and lateral flow immunoassays (LFA)[31]. Both of these methods can be sped up using a preprocessing step of immunomagnetic separation (IMS) to isolate bacteria from complex

samples[36]. It is often more effective to detect cells rather than cell metabolites, to obtain a more accurate estimate of cell count in a sample[37]. There are biosensors being developed that depend on immunological detection to produce a signal, however, it is important to refine this detection method further before applying it to biosensing platforms[36].

ELISA is one of the most widely developed assays for biochemical detection, not just foodborne pathogens, and has been developed in a way that allows for rapid testing of large numbers of samples[36]. The most effective method of ELISA for bacterial detection is a sandwich ELISA, wherein the cells or their toxins are "sandwiched" between two antibodies[31]. A primary antibody is first immobilized onto a surface, which is then exposed to a sample containing cells (ideally purified) followed by a washing step to remove anything not bound to the primary antibody[31]. Next, a solution of an enzyme-conjugated secondary antibody is added to bind to the bound cells, and washed out to remove any unbound excess, which is followed by detection via a colorimetric reaction from the bound enzymes[31]. The issues with this detection method are that it requires expensive detection equipment, and trained personnel to perform experiments[37].

LFAs rely on chromatographic-like capillary flow along a porous membrane strip rather than multiple washes of unbound particles[31]. This method uses test strips with a pore size large enough to allow cells to flow from where they are added, through a section impregnated with secondary antibody-conjugates, which are bound by the cell solution and then continue to flow along to a section with immobilized primary antibodies[37]. Detection is achieved by conjugating the secondary antibodies with gold nanoparticles, colorimetric enzymes, or another visually detectable component[36]. The advantages of LFAs over ELISAs is that they are cheaper, offer more rapid detection, and don't require trained personnel to carry out the procedure[37]. However, they have

also been shown to exhibit higher false-positive error rates than ELISA and PCR when analyzing food samples[31]. Additionally, this method of detection is more qualitative than quantitative[37].

One major drawback with both LFA and ELISA for low resource settings, is the short shelf-life and stringent storage conditions required for antibodies, which are particularly temperature-sensitive proteins, especially if they have been conjugated with enzymes[39]. Additionally, both of these methods require a pre-processing step of some form to concentrate cells or toxins from a contaminated food sample into a liquid test solution[37].

1.4.4 Nucleic Acid Testing Methods

The operating principle behind nucleic acid testing is the detection of bacterial genomic DNA (gDNA) from a contaminated sample[38]. This typically involves a lysis step to obtain the gDNA from bacterial cells, followed by capture of their genetic material and then an amplification reaction[31,37,40]. This allows for high specificity of detection, since amplification reactions can target genes that are only found in a certain bacterial species or strain of interest[36]. Bacterial enrichment is a commonly used sample preparation step, which allows for detection of bacteria at lower initial concentrations in samples by allowing them to multiply so that they are more easily detectable[36]. The methods of lysis and gDNA extraction are crucial, and typically involve the use of a centrifuge[31,40]. These methods exhibit significantly higher sensitivity and specificity of detection when compared to immunological tests[37,41].

Amplification is most commonly achieved through the polymerase chain reaction (PCR), since it provides repeatable results, can be automated, and allows for extremely high specificity via gene-targeting primers[36]. The working principle of this reaction is that double stranded DNA is exposed to thermal cycles of three temperatures which correspond to a reaction, one to denature the double helix, one to anneal primers, and one to extend and amplify a copy. The robustness of

PCR has been improved by the development of real-time or "quantitative" PCR (qPCR), which allows for real-time tracking of PCR products, and multiplex PCR (mPCR), which allows for the concurrent amplification of multiple targets[36,37,40]. An appropriate negative control would involve attempting a reaction with all the PCR components but no DNA, and should always result in no amplification unless there has been contamination of working solutions, tubes or pipette tips[40]. Both simple PCR and mPCR require the use of gel electrophoresis to detect the reaction products, whereas qPCR uses real-time fluorescence detection to track the quantities of PCR products produced in each round, which is less time consuming, provides more repeatable quantitative results, and also eliminates one potential step of sample cross-contamination[37]. These advantages of qPCR have led to the commercial development of many qPCR test kits for bacterial detection, which provide all the reagents and detailed instructions necessary, with detection limits for *E. coli* *Salmonella* of 2 x 10^2 CFU/mL without enrichment, or 1 CFU/25g after enrichment[37,42]. One downside of PCR-based detection is that it is hard to distinguish between viable and dead bacterial cells[37], however, it is worth noting that dead bacteria could have still leached foodborne toxins into the food, and thus even food contaminated with dead cells could cause illness[9]. Another challenge with PCR is that the reaction can be easily inhibited by ionic imbalances or small molecule contaminants[43,44], and thus preparing food and drink samples appropriately is imperative to PCR-based food testing.[45]

Other amplification reactions such as nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal Amplification (LAMP), and rolling circle amplification (RCA) are used as well, to combat the shortfalls of PCR[37]. These methods do not require thermal cycling and are thus even better suited to laboratories in the developing world but also point-of-care testing in low-resource medical facilities, since they do not require any major equipment to handle well-prepared

samples. Additionally, these isothermal reactions offer even better reproducibility, as it has been shown that the results from PCR-based methods can be affected by changes in thermal cycler brands and ambient room temperatures of the laboratory[40].

NASBA works by first reverse-transcribing bacterial RNA into complementary DNA (cDNA) which is then isothermally amplified using three enzymes: reverse trancriptase, T7 RNA polymerase, and RNase[37]. This reaction can distinguish between viable and dead cells through the amplification of cellular mRNA targets but is also more time consuming and less cost-effective than other nucleic acid methods[37].

LAMP makes use of *Bst* DNA Polymerase which has a high strand displacement activity, and allows for the production of large stem-loop DNA structures of varying sizes, despite the presence of large amounts of non-target DNA[42]. While LAMP is an isothermal reaction, it does require incubation at 60°C and thus would require the use of a temperature controller, albeit a cheaper piece of equipment than a thermal cycler[42]. Another downside of LAMP, is that it requires the design of four primers[42], and it is significantly harder to design these primers compared to those used for PCR[37].

Finally, RCA uses Φ29 DNA Polymerase to produce a long linear DNA strand of many repeats of the complementary sequence to a DNA circle, and this strand will stay connected to the circle until it is biochemically removed[46]. The reaction can be conducted at room temperature, and the repeated nature of the RCA products can make them massive, such that there is a noticeable change of viscosity in solutions[46], and they are easy to detect via a complementary fluorescent probe[47]. Alternatively, the repeated sequence can be used as a binding agent or a reagent in a subsequent detection reaction[46]. This can be slightly challenging since the amplification sequence

is not within the genome itself, but if the circle can be properly linked to the genome, this reaction offers the most potential for nucleic acid testing in low-resource settings.

1.4.5 Improving Sample Preparation & DNA Extraction for Nucleic Acid Testing

One of the biggest hindrances to widespread usage of nucleic acid testing, is the variability of results based on sample preparation and nucleic acid extraction[37,40,41]. Since these methods are so dependent on appropriate sample handling, bacterial enrichment, and the removal of inhibitors, it is important to validate an entire analytical procedure from start to finish to assess how the whole process would perform in a routine analysis[40]. The nucleic acid extraction step is crucial, since gDNA must be isolated as efficiently as possible, without being damaged so as to allow for an accurately quantifiable result, since each bacterial cell has a single genome copy[48]. This can be broken down further, into cell lysis and gDNA capture[40].

The typical method of cell lysis for DNA extraction involves the use of a 1:1 phenol:chloroform solution or other organic solvent to precipitate the DNA, however, it has been shown that this leaves charges and molecular salt residues on the nucleic acids that severely impact downstream reactions[49]. There have been many commercial kits, and other reagents tested to improve this DNA extraction process[50], and the best method seems to be the hot sodium hydroxide and tris (HotSHOT) reaction protocol[49], which performs much better than the conventional methods[49], and comparable or better than many commercial test kits and other extraction reagents[20, 51]. A typical HotSHOT reaction involves lysing cells in a basic solution of sodium hydroxide and EDTA at 95°C followed by neutralization with an acidic Tris-HCl solution after cooling the lysate to room temperature[49]. It is worth noting though that adding some variations to this protocol can result in higher recovery and better quality of extracted gDNA[52]. This protocol overcomes the downfalls of many other gDNA extraction methods and can be

conducted using inexpensive, common reagents found in microbiology laboratories[51], and is thus better suited to low-resource setting than many other commercial kits or special reagents[48]. However, this reaction (like many other gDNA extractions from cells) will also extract the gDNA from the food samples present[50], along with other nucleic acids like RNA, and thus treatment to isolate the gDNA of interest will result in the strongest PCR signal[48].

There have been many attempts to specifically isolate gDNA from such a reaction; However, these methods either rely on expensive equipment like a centrifuge, or charged materials such as glass fibre[53] or costly microfluidic devices[54], which cannot be directly used in amplification reactions and are not as cost effective as cellulose-paper. The one example in the literature that does use cellulose filter paper, still requires a centrifuge capable of reaching speeds above 16000 x G[55], which can be far too costly for smaller laboratories.

Thus, it would be beneficial to develop an equipment-free procedure for treatment of HotSHOT reaction products to remove small molecule contaminants and isolate the genomic DNA for downstream amplification reactions, without the need for bacterial enrichment.

1.5 Safety Standards for Bacterial Contamination of Food in Canada

The regulatory standards for bacteria, and other contaminants for that matter, in food and drink products varies among different countries and also varies according to the type of product in question. It has been well established that heat treatment, such as performed when cooking, is a good method of killing bacteria, and thus the biggest discrepancies in regulation are between products that are ready-to-eat (RTE) and those that require cooking[56]. Health Canada categorizes contaminants for each item into either a 2-class plan or a 3-class plan, depending on the nature of the food item and the contaminant in question[56]. A 2-class plan is where a product is deemed

unsatisfactory if the contaminant level surpasses the specified threshold[56]. A 3-class plan is one where there is a lower limit for marginally acceptable quality, and an upper limit beyond which the product is deemed unsatisfactory[56]. However, 3-class plans also place a limit on the number of tests that can provide a marginally acceptable result, beyond which the entire product lot becomes unacceptable[56]. This additional regulatory limit provides an added safeguard while allowing some room for manoeuvre by food manufacturers, since some products such as raw meat are likely to have some background level of innate bacteria.

E. coli falls under 3-class plans, with a marginally acceptable limit between 1.8 CFU/mL and 10^2 CFU/mL depending on the sample type, and a maximal upper limit of 10^3 CFU/mL regardless of sample type[56]. It is worth noting that lower marginally acceptable limits are typically for RTE products and these limits also often represent the marginally acceptable level of total coliform count[56]. *Salmonella* is regulated differently by Health Canada, and always follows a 2-class plan with limits of 0 CFU/mL or 1 CFU/25g, to essentially test for the absence or presence of this bacteria in a sample[56]. However, since it has already been established that many poultry and meat products will inherently contain some *Salmonella*[9], these products are then further regulated such that no more than a specific percentage of products within a lot can test positive for any species of these bacteria[56]. This qualitative test for presence or absence of *Salmonella* is consistent with the US standard as well[15].

The qualitative regulatory standards for *Salmonella* have been called into question by many experts including the EFSA, since a positive test could signify anywhere between one and millions of contaminant organisms per 25g sample with no way of determining the difference, even though the latter would pose a much bigger public health risk[15]. This becomes even more problematic when factoring in the role of *Salmonella* as transmission vectors for antibiotic resistance[O].

This qualitative metric is appropriate for RTE foods and those that are meant to be consumed raw, however it is important to develop at least a semi-quantitative method (ie. above or below a reasonable threshold that is not zero) for raw meat and poultry products[15]. Despite decreased prevalence of products that test positive for *Salmonella*[15], the number of outbreaks attributed to these bacteria has remained relatively constant[3]. There has been limited research into relative risks of different concentrations of *Salmonella*, but one meta-analysis study of outbreak data in the published literature shows that the majority of salmonellosis outbreaks (over 83%) could be traced back to a starting dosage >100 CFU, and this proportion was even higher for sever outbreaks[4]. It is hypothesized that larger contaminant doses over 100 CFU leads to clusters of cases with potential community spread, whereas lower levels lead to sporadic infection in only one or two individuals[4]. Regardless of the appropriateness of this threshold, outbreak data shows that the risk of illness due to *Salmonella* increases significantly with dose, since subjects who encountered infections with low doses became ill significantly less often[4]. Thus, there is significant need for development of at least a semi-quantitative detection method for *Salmonella* that can be applied on an industrial scale[15].

Health Canada currently employs a wide array of detection methods for bacterial contamination of food & drink samples, which are applicable for initial screenings, confirmation of detection, or both[56]. Conventional, culture-based methods are still the gold standard for confirmation tests, but the last decade has seen an increased prevalence of rapid methods used as screening assays, including many commercial kits for simple PCR & qPCR[56]. The BAX® System Standard PCR Assays are one such example that is governmentally used, with a limit of detection of 10^4 CFU/mL of *E. coli*[57] & *Salmonella*[58]. This method consists of a 10-24 hour enrichment time followed by a 3.5 hour PCR assay to achieve consistent detection at the lower limit[57,58].

1.6 Thesis Objectives

The primary goal of my thesis was to develop a rapid, low-cost method for DNA extraction from complex samples relevant to the food and drink industry, and apply it to nucleic-acid-based bacterial detection. It was important to first develop an appropriate procedure for extraction of genomic DNA from various samples that included pathogenic bacteria, in such a way that their DNA could be specifically amplified despite the potential presence of inhibitors. The specificity of detection is achieved by targeting genes that were specific to the appropriate bacteria in subsequent reactions.

The majority of the work involved DNA extraction through a modified HotSHOT protocol, followed by capture on cellulose filter paper and then amplification through PCR. The HotSHOT extraction procedure was chosen since it is quick, it involves simple reagents available at any microbiology laboratory, and it does not interfere with downstream processing of extracted DNA. Cellulose filter paper was selected as the platform of choice for DNA capture since it is a very cheap, porous material that allows for passive filtration, and it is easy to modify with wax printing. PCR was selected as the amplification method since it is highly specific, presents reliable results, and is well established in methods of bacterial detection. These experiments are presented in Chapter 2.

Alongside this work, a few other extraction methods, capture platforms, and amplification procedures were tested, with varying degrees of success. Most notable of these was the adaptation to this method of coupling a HotSHOT lysis and gDNA extraction onto cellulose paper with RCA instead of PCR as an amplification step. The combination of this method allowed us to detect the presence of 10^5 cells, which provides proof-of concept that we can perform DNA extraction and amplification without thermal regulation, which is a crucial step towards developing a reliable,

low-cost technique for efficient, rapid detection of bacterial contamination in resource-limited settings. These experiments are covered in Chapter 3.

Overall, this thesis aims to demonstrate the presented reaction set as a way to semi-quantitatively assess bacterial contamination in a variety of vectors without bacterial enrichment, which is a step towards novel biosensing platforms that can optimize detection and food safety procedures for the food and drink industry. This is specifically achieved via a modified HotSHOT lysis reaction that preserves the genomic material followed by gDNA isolation on cellulose filter paper and finally an amplification reaction that targets and amplifies a specified gene to produce a detectable signal.

Chapter 2: Bacterial Detection in Food & Drink Samples via Isolation & Amplification of Genomic DNA on Nitrocellulose Filter Paper

2.1 Introduction

As mentioned previously, the HotSHOT extraction protocol is advantageous for cell lysis with the intent to extract gDNA, since this protocol is faster than other typical gDNA extractions and produces a higher yield of gDNA that is also of better quality[50], which makes it easier to use in downstream reactions[59]. Furthermore, the required reagents are much cheaper and have significantly lower toxicity to humans and the environment[48], which makes them more well suited to low-resource diagnostic applications.

One downside of performing a HotSHOT extraction in the context of detecting bacteria in food and drink samples is that the reagents will also extract gDNA from any other organisms in the sample, including the food itself[20,52]. Additionally, the reaction products contain a mixture of gDNA and other cellular components which may interfere with downstream reactions, such as RNA which has been shown to interfere with PCR[59]. Thus, it is useful to develop a method to efficiently isolate this gDNA before subsequently amplifying it to create a more specific, detectable signal.

There have been many recent developments in paper-based nucleic acid tests which are simple, portable, and low cost, and are thus well suited to low-resource diagnostics[60-62]. Past porous material-based extraction has typically been performed with charged materials such as glass fibre, chemically modified paper, or costly microfluidic devices. These materials are less cost effective than cellulose and may not be compatible with the current target and signal amplification methods applied to cellulose and nitrocellulose paper[53,54]. Recent research has proven that

Whatman Grade 1 filter paper can capture and retain DNA during a brief washing step. This paper can then directly be used directly in a DNA amplification step with relatively high efficiency[63]. There has also been recent research into using unmodified cellulose paper discs as a cost-effective method to recharge spin columns for gDNA extraction. This has shown promising results with a high extraction efficiency; however, the spin-column method is more time consuming and requires the usage of a centrifuge capable of reaching speeds above 16000 x G [55].

Researchers in our lab group at McMaster University have drawn inspiration from this and developed a procedure for purification of gDNA on untreated cellulose paper using a modified HotSHOT extraction method. Our method can also be performed with minimal laboratory equipment while using standard chemicals and materials that would be found in a typical biochemistry laboratory. It is also able to effectively separate out other nucleic acids and any large cell debris or other contaminants from the gDNA of interest without the use of expensive solvents or equipment. This development of a cellulose filter-based DNA extraction method has enormous potential for integration with existing PoC nucleic acid-based assays, achieving sample preparation, nucleic acid amplification, and detection in a single paper device. A quick overview of how the method works can be seen in Figure 2.1 with further experimental details expanded on in Section 2.2.

Preliminary tests with this method have shown that it is effective at retaining gDNA on paper whereas other cellular components and RNA can be washed out. The gDNA can then be recovered from the paper with an adequate yield to produce an amplification signal through PCR. This process eliminates lengthy sample preparation steps required for nucleic acid testing.

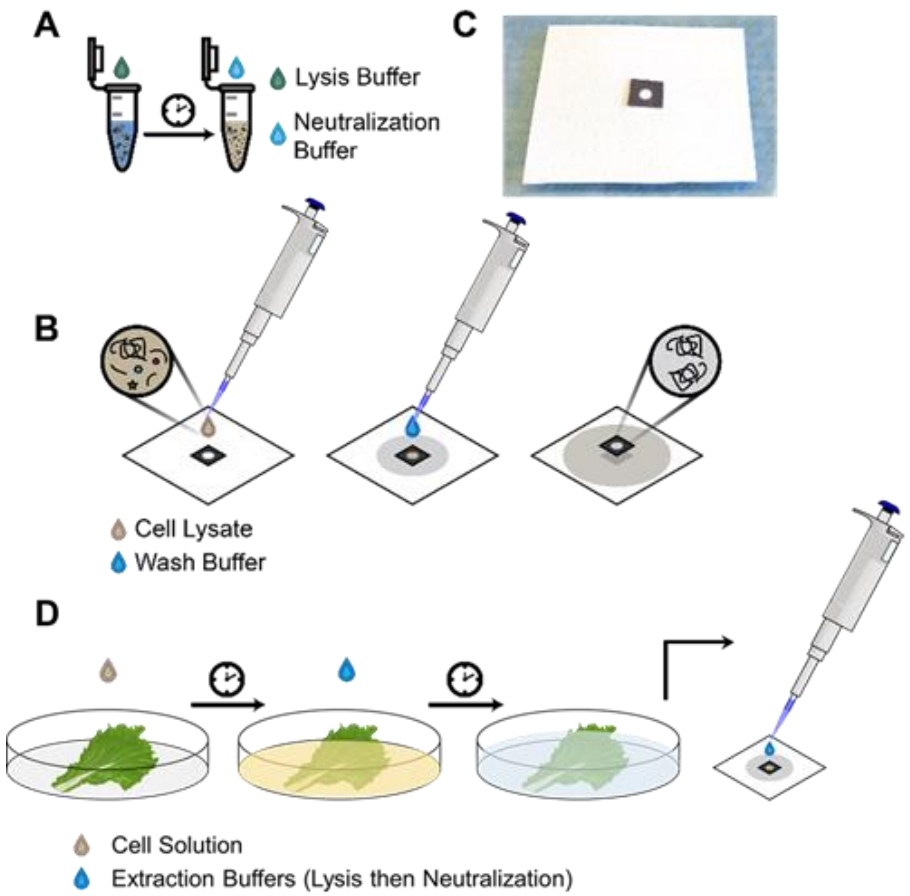


Figure 2.1 Diagram of paper-based gDNA extraction method. A) Modified HotSHOT lysis of bacterial cells to extract DNA. B) Paper extraction and purification method with wash. C) Image of assembled paper-based extraction device. D) Preparation, incubation, and lysis of food samples to be used for extraction. In all of these cases, the gDNA is eluted from the filter before being used in PCR.

The aim of my work is to demonstrate that the proposed low-cost method can detect bacteria from solid food samples and to calculate an estimate for the lower limit of detection in free solution as well as complex in solid & liquid samples. Additionally, I performed tests with *Salmonella* Newport in addition to *E. coli* to prove that this method is versatile and works with different types of bacteria, so long as the appropriate primers are chosen for PCR. Primers chosen had been previously shown to effectively detect *E. coli* & *Salmonella* through PCR[64,65]

2.2 Materials & Methods

2.2.1 Materials & Equipment Used

Native Taq Polymerase (which was shipped with 10X Taq Polymerase Buffer and 50mM $MgCl_2$), GeneRuler Low Range DNA Ladder (for nucleotides 25-700 bp in length), BSA (40mg/mL) and a 10mM dNTP mix were purchased from Thermofisher Scientific. PCR primers were all ordered from Integrated DNA Technologies®, with sequences shown in Table 2.1. GelRed® was added to all agarose gels to stain DNA, and was purchased from Biotium. All other chemicals were ordered from Millipore Sigma and diluted in house, unless otherwise specified. Paper micro-well plates were printed using a Xerox ColorQube 8570N solid wax printer. All gel images were captured on a BioRad ChemiDoc XRS+ with the setting "GelRed Nucleic Acid Gels"

Table 2.1 Sequences of PCR Primers Used to Target and Amplify Bacterial gDNA

Gene Target	Abbreviation	Sequence (5'-3')
E. coli ybhJ-C (Forward End)	**ECFP**	AGA GCG CGA GAT TAT CAA GG
E. coli ybhJ-C (Reverse End)	**ECRP**	TGC AGA GGC GAA GAA GTA AG
S. Newport *sipB-C* (Forward End)	**SNFP**	ACA GCA AAA TGC GGA TGC TT
S. Newport *sipB-C* (Reverse End)	**SNRP**	GCG CGC TCA GTG TAG GAC TC

2.2.2. Paper Device Fabrication & Extraction Method

Paper waste pads were prepared from sheets of Whatman Grade 1 filter paper, cut into squares measuring 5cm by 5cm. Filters used for DNA extraction were prepared by wax printing a 96-microzone pattern onto sheets of Whatman Grade 1 filter paper. Zones were printed with hole sizes of 4mm, and then individual filters were cut out such that each filter had one well with wax barrier measuring approximately 8mm x 8mm. The extraction device was assembled by placing a cut filter

paper onto a waste pad, such that the printed wax side faced upwards. *E. coli* samples were processed by conducting lysis as previously described. An appropriate amount of PBS buffer was added to each sample to bring the volume to 100µL. Each sample was pipetted slowly onto the unprinted area on the filter paper, such that the droplet did not overflow onto the wax printed region. After sample had soaked completely through into the waste pad, washing was done by adding 100µL of PBS buffer onto the filter paper in a similar fashion and waiting for it to flow through (Fig. 2.1B, 2.1C). The filter paper was then removed with tweezers and further processed through elution before PCR

2.2.3 Elution of Genomic DNA from Paper Filters

After extraction via filter method, paper filters were placed in a 0.2mL Eppendorf tube. 45µL of appropriate elution buffer, which was typically water unless otherwise stated, was added such that the paper filter was completely submerged. Tubes were vortexed vigorously for at least 10 seconds. 35µL of resulting solution was removed and used directly for PCR. This step was added rather than directly adding the filters to PCR as it was determined that direct addition of the filters to PCR caused some inhibition of amplification (potentially due to the printed was), whereas PCR with eluted DNA from the filters gave a much better amplification signal.

2.2.4 Cell Lysis via Modified HotSHOT Method

2x HotSHOT+Tween buffer was prepared with the following reagent concentrations: 50mM NaOH, 0.4mM EDTA, 0.1% Tween-20. 2x Neutralization Solution was prepared with the following reagent concentrations: 50mM HCl, 10mM Tris-HCl. Both solutions were sterilized by passing them through a dead-end 0.2µm filter.

Lysis was performed by adding 5µL of prepared cell culture or negative sample to 10µL of 2x HotSHOT buffer. Samples were incubated at room temperature, rather than 65°C, for 10

minutes (Fig. 2.1A).[50] Lysis at 65°C was briefly tested as well, but resulted in decreased signal in downstream PCR-based testing. After incubation, 10µL of 2x Neutralization buffer was added and the solution was mixed by pipetting to homogenize the solution before filtration.

2.2.5 Cell Culture Preparation

E. coli cell culture was prepared by inoculating 5mL of TSB media with 100µL of previous culture, and allowing overnight growth at 37°C, until OD600 reached approximately 1.0. This was then split into 1mL samples of culture, which were centrifuged at 8000 x G for 3 minutes, then the supernatant was removed and 1000µL of PBS buffer was added. Centrifugation and resuspension was repeated twice more, discarding PBS buffer supernatant each time, before finally resuspending cells in PBS buffer. Cell concentration was quantified through OD600 readings. A stock *E. coli* sample was prepared from this sample with a concentration of 2×10^7 cfu/mL, or 10^5 cfu/5µL, which is the concentration used in all experiments unless otherwise stated. Cell concentrations were tested and confirmed by plate counts. For the experiments with growing bacteria on food samples, *S.* Newport was grown and washed with PBS in the same way.

2.2.6 Polymerase Chain Reaction Protocol

All PCR reactions were performed in a reaction volume of 50µL, with reagents added as specified in Table 2.2. Note that it is important to use native Taq polymerase, as it was determined that recombinant Taq may cause false positives especially with *E. coli*. PCR for both targets was run with a 10 minute initial denaturation time at 95°C, followed by 30 cycles consisting of a 30 second denaturation at 95°C, a 30 second annealing time at 52°C, and a 30 second extension at 72°C, ending with a 5 minute final extension at 72°C. PCR products were then cooled to 4°C before 10µL from each 50µL reaction was run through an agarose gel with a concentration of 1X GelRed™ dye for DNA detection.

Table 2.2 Quantities and Final Concentrations of Each Reagent Added to a Typical PCR

Reagent	Volume Added	Final Concentration
dNTP Mix (2mM)	5.0 µL	200µM
10X *Taq* Polymerase Buffer	5.0 µL	1X
BSA (40 mg/mL)	1.0 µL	0.8mg/mL
50mM $MgCl_2$	2.0 µL	2mM
Native *Taq* Polymerase	0.2 µL	1 unit/ 50µL
Foward primer (25µM)	1.0 µL	0.5µM
Reverse primer (25µM)	1.0 µL	0.5µM
Sample + H2O	34.8 µL	N/A

2.2.7 Preparation & Processing of Environmental & Food Samples:

Soil Sample: Soil samples were prepared by collecting soil from outside the laboratory, then adding sufficient water to create a 20% w/v sample. Spiked sample was prepared by adding appropriate amount of previously prepared *E. coli*, suspended in PBS buffer, such that final concentration was 2×10^7 cfu/mL. Prior to use, samples were vortexed vigorously, then allowed to settle for 1 minute to prevent larger particulate matter from clogging the filter.

Skim Milk Sample: Skim milk was prepared from powdered skim milk, as per manufacturer directions. Samples were spiked in a similar manner to spiked soil samples, described previously.

Lettuce Sample: Lettuce samples were prepared by first cutting a head of romaine lettuce into 7.5cm segments. Two leaf segments were placed into a 100mm x 20mm petri dish, and then 5mL of bacteria suspended in PBS buffer with a concentration of 2×10^7 cfu/mL (*E. coli* or *S.* Newport) was spread all over the dish and well mixed. For limit of detection tests, a serial dilution of the cells was added in the previous step. Dishes were refrigerated at 4°C for 10 hours. Prior to lysis, negative and positive samples were brought to room temperature and swirled vigorously to mix.

Chicken Sample: Chicken samples were prepared by first cutting boneless, skinless chicken breasts into roughly 4cm by 4cm by 1.5cm pieces. One piece of chicken was placed into a 100mm

x 20mm petri dish, and then 5mL of bacteria suspended in PBS buffer with a concentration of 2×10^7 cfu/mL (*E. coli* or *S.* Newport) was spread all over the dish and well mixed. For limit of detection tests, a serial dilution of the cells was added in the previous step. Dishes were refrigerated at 4°C for 10 hours. Prior to lysis, negative and positive samples were brought to room temperature and then swirled vigorously to mix.

2.2.8 Lysis for Spiked Liquid Samples

Lysis was performed by adding 5µL of prepared liquid samples to 10µL of 2x HotSHOT buffer, followed by incubating at room temperature for 10 minutes. After incubation, 10µL of 2x Neutralization buffer was added.

2.2.9 Lysis for Spiked Solid Food Samples

Lysis was performed by adding 10mL of 2x HotSHOT buffer to the dish for each sample and swirled vigorously to mix, before incubating at room temperature for 10 minutes. After incubation, 10mL of 2x Neutralization buffer was added and dishes were swirled vigorously to mix (Fig. 2.1D). 25µL of the resulting liquid was then combined with 75µL of PBS and this was then to carry out a paper extraction, as previously described.

2.2.10 PCR with Spiked Samples

Samples that were processed via paper filtration method were first lysed using the modified HotSHOT method, then extracted via paper filtration, both as previously described above. DNA was then eluted from filters and used in PCR as before. When using unextracted samples for PCR, 25µL of environmental or food sample after the HotSHOT reaction was used in all cases. This 25µL of sample was added directly to PCR mixture, along with 10µL of water, in place of the 35µL from paper filtration and elution.

2.2.11 Limit of Detection PCR Tests

Cell culture stocks with a concentration of 2×10^7 cfu/mL were prepared and washed with PBS as previously described. These were then serially diluted 10-fold at a time by preparing tubes with 900µL of PBS and then mixing in 100µL of stock solution or a previous dilution. Spiked samples were prepared as previously described, except for the use of serially diluted cell solutions in place of the stock of 2×10^7 cfu/mL. Samples were first lysed using the modified HotSHOT method, then extracted via paper filtration, both as previously described above for the respective sample type. DNA was then eluted from filters and used in PCR as before. All LoD tests used the primers targeting the *ybhJ-C* gene for *E. coli* and the *sipB-C* gene for *Salmonella* Newport.

2.3 Results and Discussion

2.3.1 DNA Extraction & Proof of PCR with Complex Liquids

Previous work had shown that the proposed method was effective at detecting *E. coli* from a cellular suspension as well as more complex liquids such as milk or a soil slurry. These liquids were chosen for their known tendencies to inhibit PCR and thus the first set of experiments was to replicate these tests to verify the previous results. A set of 3 spiked 1 mL samples each for soil and milk were prepared to have a final concentration of 2×10^7 cfu/mL and then 5 µL at a time was used for each extraction. Controls were designed such that the same lysis, extraction, and amplification procedure was carried out, but with pure milk or soil slurry that had not been spiked with bacteria. Additionally, another set of lyses was performed, and the lysate was added directly to PCR without the paper extraction step to explore the effect that this step has. An agarose gel electrophoresis was conducted after PCR and the results can be seen in Figure 2.2 below. It is apparent that PCR was completed inhibited for lysed samples that did not undergo the paper extraction, which demonstrates that this is a key step in the procedure to produce an appropriate

amplification signal. Thus, this DNA isolation method is effective at producing a clear signal when detecting 10^5 cfu of *E. coli* in PCR for milk and liquid soil slurries that were spiked with bacteria, despite both of these vectors being known as highly inhibitive to PCR[43,44].

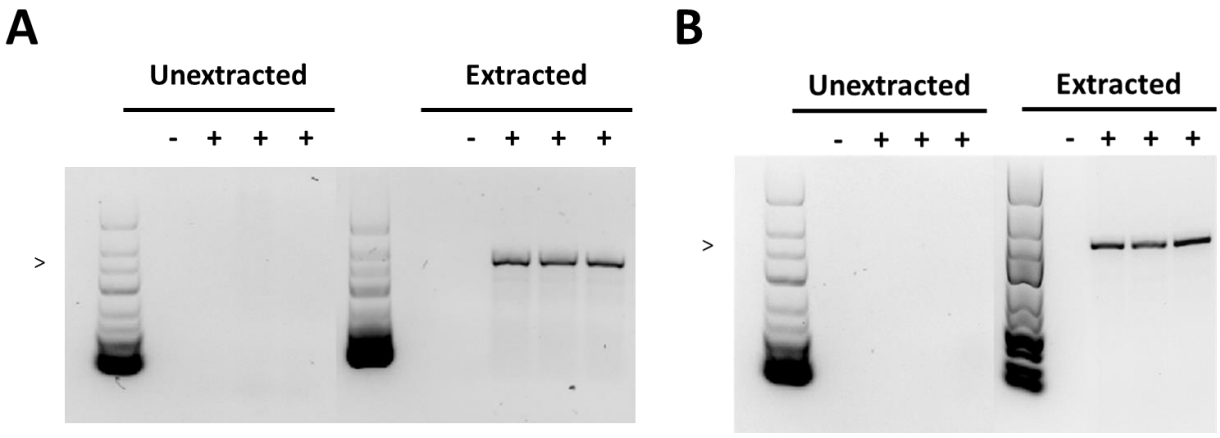


Figure 2.2 PCR amplicon of complex, inhibitory liquids, namely A) 20% w/v soil and B) skim milk. Samples marked with a '+' were spiked with E.coli whereas those with a '-' were not. Samples were either added directly as-is to PCR reaction or extracted through paper-based method and eluted for use in PCR reaction. PCR products were analysed through agarose gel electrophoresis.

2.3.2 DNA Extraction & Proof of PCR with Food Samples

Since it was proven that bacterial gDNA could be extracted, purified, amplified and detected from a highly inhibited liquid sample, the next application-based experiment was to evaluate if the method could be used to extract and purify gDNA from contaminated solid food samples and then confirm whether the purity and yield would produce an adequate amplification signal. For these experiments, a second type of bacteria, *Salmonella enterica* serovar 'Newport' (SN), was tested in addition to *E. coli* K12 (ECK12) since *Salmonella* is a common contaminant of food products, and we wanted to prove the versatility of this extraction method to detect any bacteria, so long as the appropriate primers to target its genome for PCR were selected. The two types of food samples chosen were chicken breasts and romaine lettuce, since both have been the subject of many product recalls due to contamination and both have been shown to cause inhibition in PCR[66,67].

Additionally, a set of PCR reactions was prepared with 25µL of food sample after the HotSHOT reaction, containing 10^5 cfu, directly added without extraction, to compare the degree of inhibition with and without extraction. Samples were analyzed on a 2% agarose gel, and all gels are shown in Figure 2.3 below.

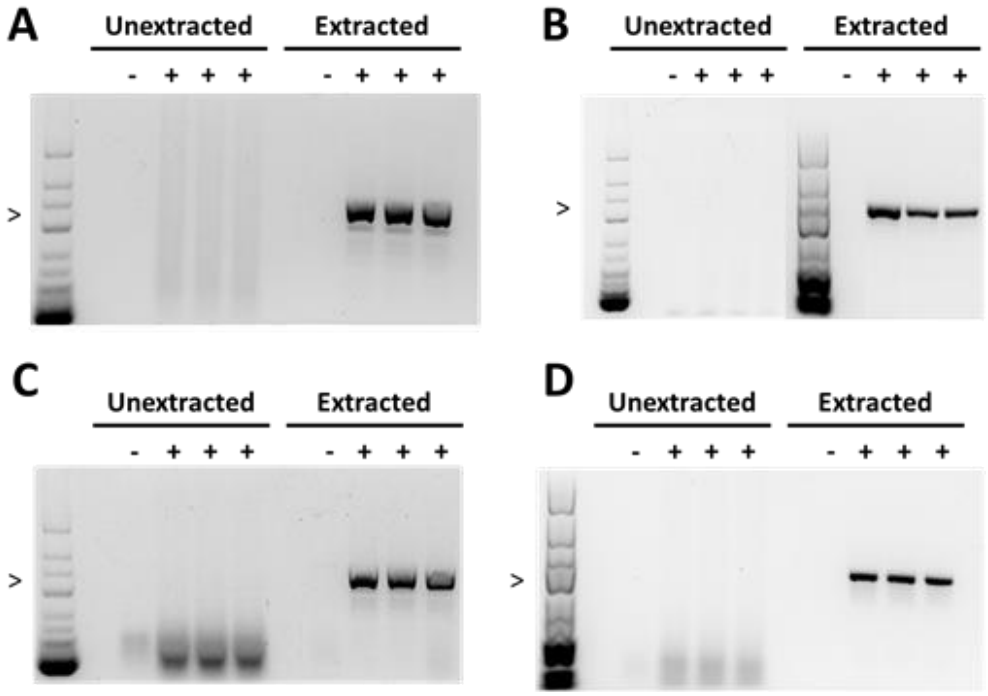


Figure 2.3 PCR amplicon of food samples, namely A) romaine lettuce spiked with *E. coli*, B) romaine lettuce spiked with *S.* Newport, C) chicken breast spiked with *E. coli*, and D) chicken breast spiked with *S.* Newport. PCR products were analysed through agarose gel electrophoresis. Samples were either added directly as-is to PCR reaction or extracted through paper-based method and eluted for use in PCR reaction. PCR products were analysed through agarose gel electrophoresis.

For all four combinations of bacteria and food, adding the lysate directly to PCR led to complete inhibition of PCR. However, samples which had undergone the paper-based extraction showed strong bands (Fig. 2.3). A large amount of particulates was visible in all of the HotSHOT reaction products for chicken breast; however, these did not seem to cause inhibition of PCR, likely due to the paper extraction step in our procedure. It is hypothesized that the dark bands in the

unextracted chicken samples for both *E. coli* and *Salmonella* represent primer-dimers formed in PCR due to their small size of around 25-50 base pairs (Fig 2.3 C,D).

2.3.3 Determining the Limit of Detection of Bacterial Cells in Solution

After proving that 10^5 cells could be detected in each of the cases above as well as the prior proof of concept tests, each test was repeated with six serial 10X dilutions to determine the limit of detection (LoD), which is the lower limit for the number of cells that could be consistently detected and distinguished from background signal.[68] The experimental procedure was the same as before with the exception of using diluted samples. PCR band intensities were quantified using the "Analyse:Gels" function within ImageJ (V1.51) software. Band intensities were measured as a percentage of the brightest band in the image (50 bp fragment in the GeneRuler Low Resolution (LR) DNA ladder) and then normalized as a percentage of the intensity of the undiluted sample's band (ie the proven sample with 10^5 cfu). After normalization, the intensities from repeats of the same experiment were averaged, and the standard deviation as used to produce error bars. A band was qualified as detected if it was visible on a gel image and had an intensity with error bars that did not cross 0.

Before determining LoD, it is first important to determine the limit of blank (LoB), which is the highest apparent number of cells detected in replicates of blank samples.[68] The LoB and LoD can be estimated from the mean and standard deviation using Equations 1 and 2 below, where c_β is the relevant test statistic for each sample type, and the "low concentration sample" was selected as the highest dilution of that sample that would still reliably produce bands on a gel after PCR. The test statistic is typically 1.645 which represents a one-tail test from a normal distribution for the lower limit with 95% confidence.[69] However, this only applies when there are more than 60 samples, and since there were less samples tested in our experiments, the test statistics came from

Student's t distribution at the same level of confidence. The degrees of freedom used was the difference between the number of samples created and the number of gels analysed for each sample type. The calculated limits were converted into a quantity of cells per PCR reaction using a calibration curve that accounted for the relative pixel intensity across all gels of the same sample type. Calibration curves and all gel images are provided in Appendix A.

$$LoB = Mean_{blank} + c_{\beta} SD_{blank} \quad (1)$$

$$LoD = LoB + c_{\beta} SD_{low\ concentration\ sample} \quad (2)$$

The first experiment was to determine the LoD of cell solutions directly added to PCR as compared to the LoD for cell solutions that have undergone the paper extraction method. It was determined that down to 43 cells (8.6 x 10^3 CFU/mL) of *E.coli* could be detected through PCR without extraction, but with the extraction this decreased to 37 cells (7.4 x 10^3 CFU/mL), with the bands on the gel also appearing stronger. A similar test run with *S.* Newport demonstrated a LoD of 59 cells (1.2 x 10^4 CFU/mL) without extraction, and a decrease to 45 cells (9.0 x 10^3 CFU/mL) with extraction, and the bands on the gel appeared cleaner after extraction. These results indicate that a higher proportion of the present cells were detected after extraction. Figure 2.4 displays the average relative signal measured from each dilution of cells. It appears that the variance is smaller for lower cell concentrations after extraction, indicating more consistent detection for fewer cells, however the statistical significance of the reduced variance could not be verified due to the low sample number. It is also worth noting that since only 10µL from each 50µL PCR reaction was used for gel electrophoresis that this LoD is potentially higher and thus a conservative estimate of the true LoD which could be confirmed through qPCR.

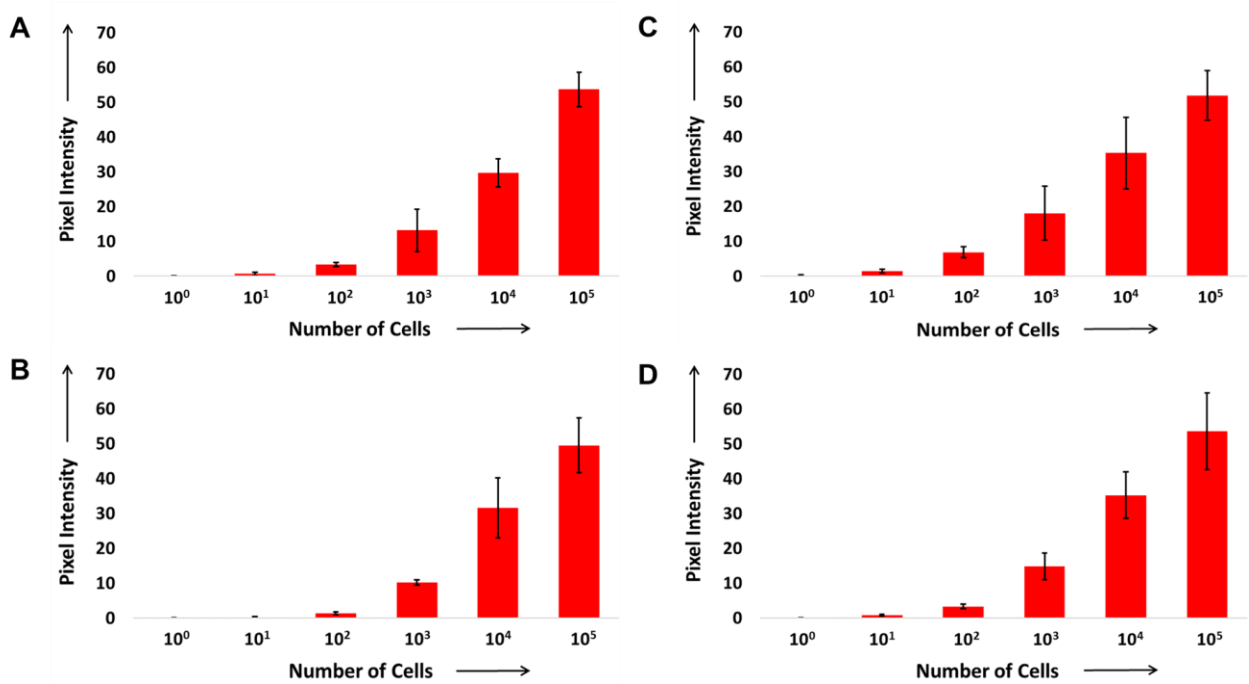


Figure 2.4 Bar charts of the average signal intensity of PCR products for: (A) E.coli cells added directly to PCR (B) E.coli cells extracted with the modified HotSHOT protocol before being added to PCR (C) S. newport cells added directly to PCR (D) S. newport cells extracted with the modified HotSHOT protocol before being added to PCR. The error bars represent one standard deviation.

2.3.4 Determining the Limit of Detection of Bacterial Cells in Complex Samples

The next experiments involved determining the LoD for each environmental sample. For the soil samples we found that it was possible to detect 640 cells (1.3×10^5 CFU/mL) of *E. coli*. However, the milk samples appeared to be more inhibited, since only 92 cells (1.8×10^4 CFU/mL) of *E. coli* could be detected.

The final set of experiments was to determine the LoD for all combinations on both bacteria tested on both food samples. It is clear that detection with food samples works much better for *S.* Newport with an LoD of 66 cells (1.3×10^4 CFU/mL) on romaine lettuce, and 200 cells (4.0×10^4 CFU/mL) on chicken. Detection of *E. coli* grown on food worked well on the romaine lettuce samples, with an estimated LoD of 137 cells (2.7×10^4 CFU/mL). However, the chicken breast samples exhibited significantly less amplification for E. coli, with an estimated LoD of 1023 cells

(2.0×10^5 CFU/mL), a whole order of magnitude higher than any of the other calculated LoDs except for the soil slurry. It is also worth noting that since only 10µL from each 50µL reaction was used for gel electrophoresis that this LoD is potentially higher than the actual LoD which could be confirmed through qPCR. It is unclear whether this much worse detection limit is due to inhibition from the chicken breast or an inability of *E. coli* cells to be grown on and extracted from chicken breast tissue, since these bacteria typically live in the instestines.

Figure 2.5 displays the mean average relative signal measured from each dilution of each environmental/food sample, whereas the numerically estimated LoDs for all samples are summarized in Table 2.3 below. It is worth noting that these LoDs are comparable to that of the BAX® System Standard PCR Assays (10^4 CFU/mL) which has been governmentally approved for bacterial screening.

Table 2.3 Summary of the estimated limits of detection of bacterial cells in each of the different sample types tested by simple PCR followed by agarose gel electrophoresis

Sample Type	**Limit of Detection**	
	CFU/PCR reaction	**CFU/mL**
Unlysed *E. coli* in solution	43	8.6×10^3
Lysed *E. coli* in solution	37	7.4×10^3
E. coli in milk	92	1.8×10^4
E. coli in soil solution	640	1.3×10^5
E. coli on chicken	1023	2.0×10^5
E. coli on lettuce	137	2.7×10^4
Unlysed *Salmonella* in solution	59	1.2×10^4
Lysed *Salmonella* in solution	45	9.0×10^3
Salmonella on chicken	200	4.0×10^4
Salmonella on lettuce	66	1.3×10^4

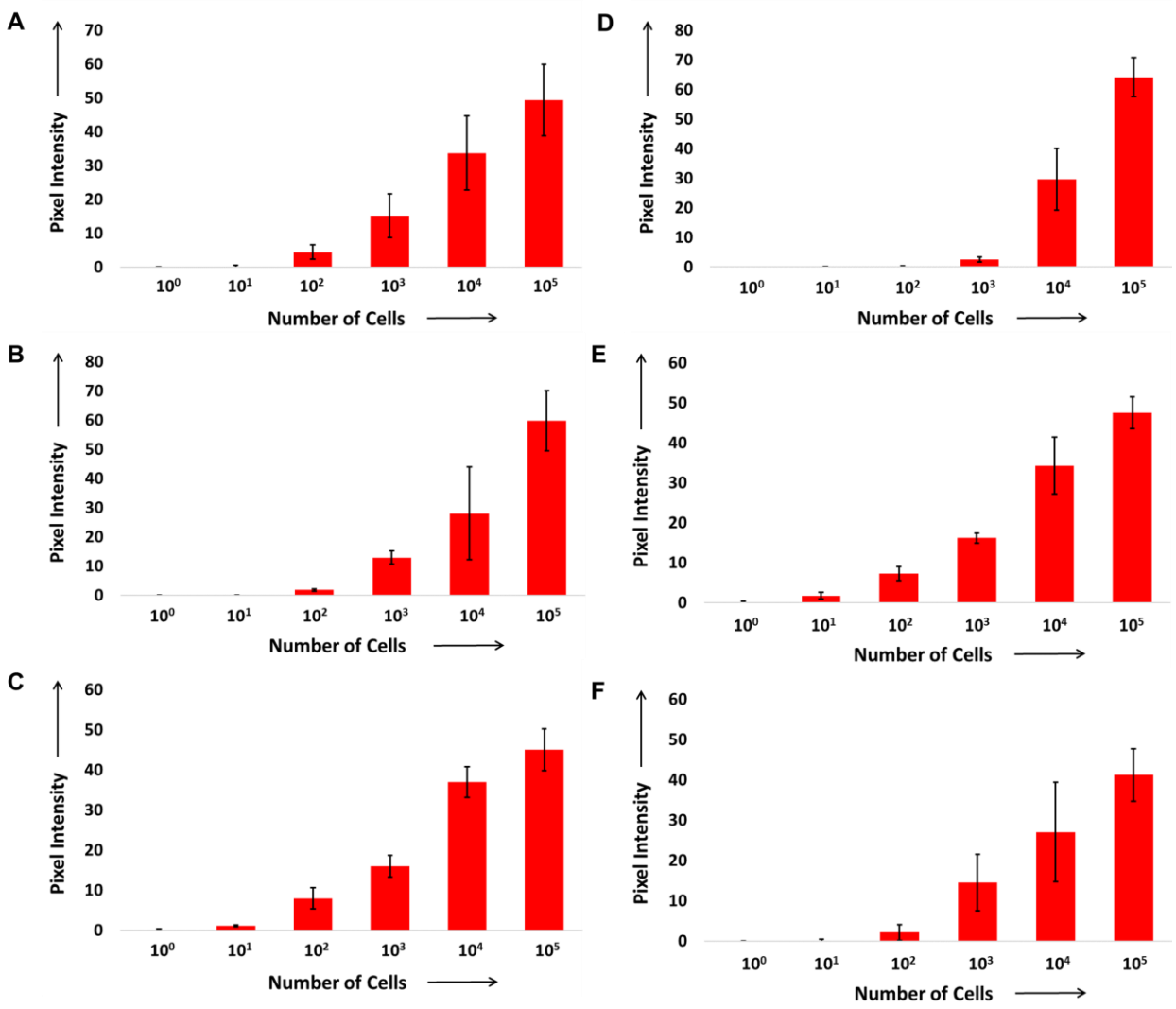


Figure 2.5 Bar charts of the average signal intensity of PCR products for: (A) *E. coli* cells added to a 20% w/v soil slurry (B) *E. coli* added to skim milk (C) *E. coli* cells added to romaine lettuce (D) *E. coli* cells added to chicken breast meat (E) *S.* Newport cells added to romaine lettuce PCR (F) *S.* Newport cells added to chicken breast meat. The error bars represent one standard deviation.

2.4 Conclusions

Generic cellulose filter paper with a porosity (and thus particle retention limit) of 11 µm shows promise as an efficient material for retention of gDNA from mixed cell lysate solutions, even after washing in a high salt buffer. These results demonstrate that the modified HotSHOT lysis coupled with a cellulose filter paper extraction and PCR amplification is a quick, cheap and effective method to detect bacterial gDNA in complex solid and liquid samples without bacterial

enrichment. This procedure is able to eliminate small molecule contaminants so much so that even completely inhibited PCR reactions can be recovered to the point of detecting hundreds of cells. Even with multiple organisms in one sample, this method is effective at isolating and amplifying the gDNA of interest through targeted primers for PCR. This procedure was applied with diminishing numbers of cells to determine an estimate for the lower limit of detection for each sample type. The presented limits of detection showcase the power of this extraction and simple PCR technique as a semi-quantitative rapid method of detection, with limits similar to that of the BAX® System Standard PCR Assays without the need for bacterial enrichment. Moving forwards, it would be beneficial to further explore the quantifiability of this method so that it can be used to determine an accurate estimate for cell count from a sample, through incorporation of qPCR.

Chapter 3: Attempted Alternative Procedures

3.1 Capture of bacterial gDNA onto agarose beads

Bead-based capture systems have long been reported for isolation of DNA from a cell lysis reaction, since beads settle quickly and allow for DNA extraction without centrifugation[40]. As such, there were attempts made to extract bacterial gDNA onto streptavidin-coated beads, via a linker sequence that has one end complementary to a gene of interest and a biotin molecule on the other end. This capture method takes advantage of the well-documented interaction between biotin and streptavidin, which form one of the strongest bonds found in nature[70]. A schematic of the capture and linkage method is shown in Figure 3.1 below. Two different types of streptavidin-coated beads were tested, agarose beads and ferric-based magnetic beads. The biotinylated capture sequence used was: `5'-/5Biosg/TTTTTTTTTTTTTTTTTTTTCAACTCTGGCTCCGTCTCTG-3'`

E. coli cell cultures were prepared as mentioned in Section 2.2.5 and diluted to a final concentration of 10^6 cfu/mL, before being centrifuged and decanted to create a pellet of cells. A similar modified HotSHOT lysis was performed with 500µL of 2x HotSHOT buffer added to the pellet and mixed, along with 1µL of a 2.5µM solution of the biotinylated linker sequence. This mixture was incubated at room temperature for 10 minutes before 500µL of 2x Neutralization buffer was added and mixed by pipetting. Finally, 10µL of a 50% slurry of beads was added immediately after neutralization, and tubes were left to rotate for an hour at room temperature for the biotin-streptavidin linkage to form. The beads were then allowed to settle, decanted of the liquid, and washed 3 times with 1000µL of PBS buffer by shaking the tubes, allowing the beads to settle and decanting the PBS. After the third wash, the beads were resuspended in 35µL of H_2O and this was directly added to a PCR with the same reaction conditions described in Section 2.2.6 and Table 2.2.

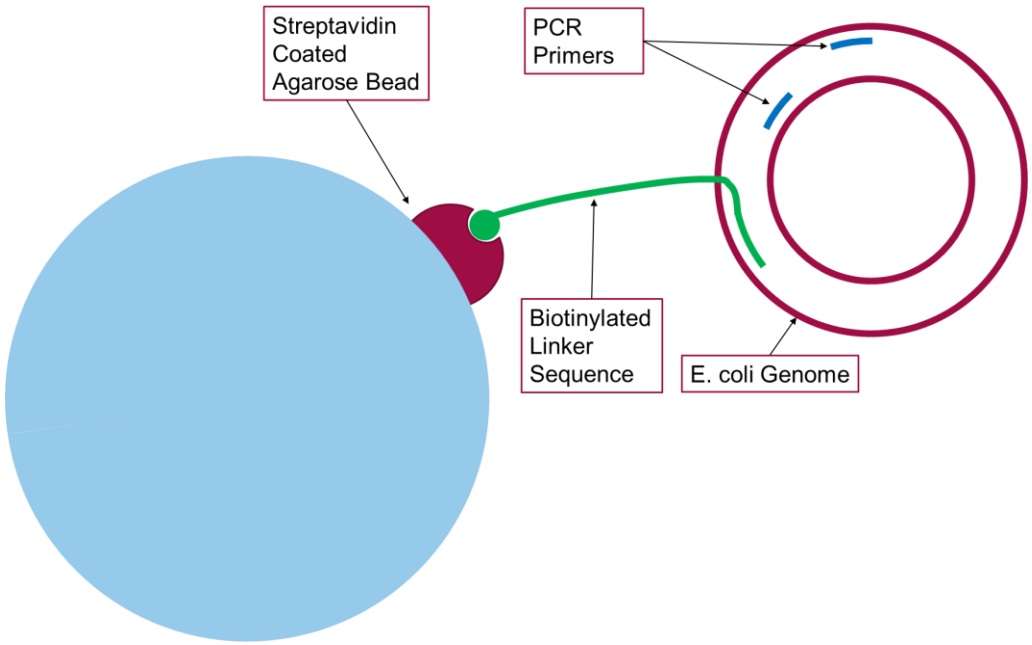


Figure 3.1 Schematic representation of the agarose bead capture system for bacterial gDNA. This image is not to scale.

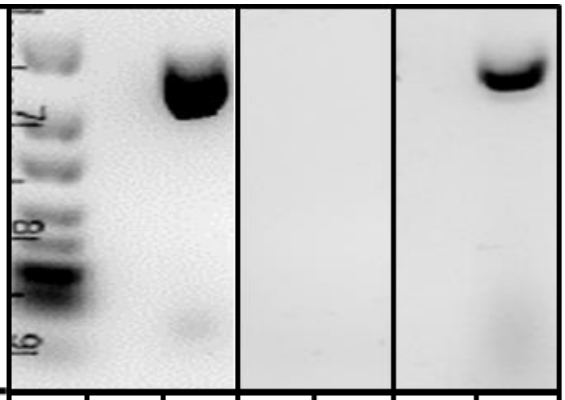
The first proof-of-concept experiment required for this extraction system was to detect that gDNA could be effectively captured onto beads. This was achieved by a simple test of the extraction procedure, selectively excluding the biotinylated linker and or bacterial cells. The detection of gDNA on beads was achieved using a 1X concentration of GelRed™ DNA dye.

Table 3.1 Proof-of-concept test to show that gDNA can be immobilized on streptavidin-coated agarose beads using a biotinylated linker sequence that is complementary to part of the genome.

	No Biotinylated Linker	With Biotinylated Linker
No Cells		
With Cells		

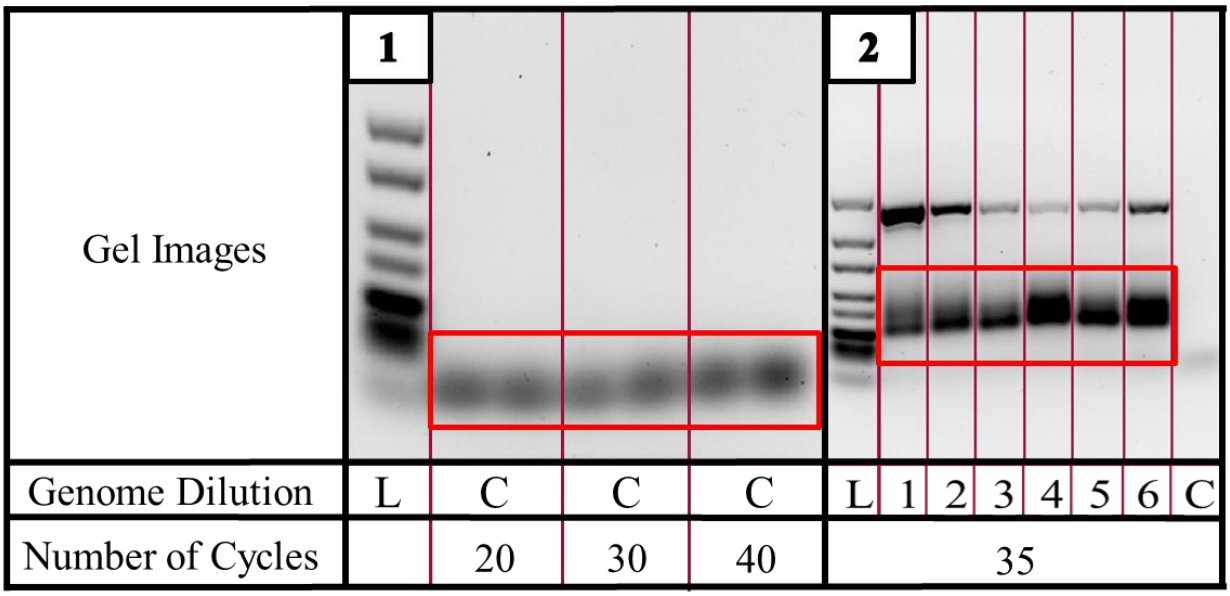
The next proof-of-concept test was to determine whether 10^6 bacterial cells could be detected via a 20-cycle PCR, which was tested by comparing cells directly added to PCR in solution with the same number of cells extracted using both magnetic and agarose beads. All 3 tests involved a control with the same procedure but no cells. The 3 gels were summarized into Table 3.2 below.

Table 3.2 Proof-of-concept test to evaluate whether 10^6 cells could be detected through extraction on beads followed by PCR. L – Ladder ; C – Control ; S - Sample

Gel Image							
Lane	L	C	S	C	S	C	S
Sample Type	Cells in solution			Magnetic Beads		Agarose Beads	

It appears that a magnetic bead extraction was not conducive to detection via PCR, and an agarose bead extraction resulted in significantly less signal from PCR than adding in cells directly. However, this experiment does show proof that an agarose bead-based extraction procedure was capable of capturing gDNA in a manner that allowed for DNA amplification. It is notable that aside from the gene of interest, there was some nonspecific amplification present when PCR worked. The next important step was to determine a LoD for bacterial cells in solution through an agarose bead gDNA extraction followed by PCR. This was done using dilutions of a 2×10^7 cfu/mL solution of bacterial cells in PBS. Since we had already observed detection for 10^6 cells, the 10X serial dilutions 1-6 were extracted by the same protocol followed by a 35-cycle PCR. Negative controls of 20, 30 and 40 cycles were also tested. These gels are shown in Table 3.3.

Table 3.3 Gel electrophoresis images of PCR tests following an agarose bead extraction, with non specific amplification marked in red boxes. Panel 1 shows a negative control test with varying numbers of cycles. Panel 2 shows an attempted LoD test for 10^6 down to 10^1 cells with samples subjected to an agarose bead extraction followed by a 35-cycle PCR. L – Ladder ; C – Control



These results were not expected, as the negative controls showed some non-specific amplification, and the samples showed two bands of nonspecific amplification. It was hypothesized that the PCR primers were interacting with the agarose beads and becoming entrapped at high temperatures, since the controls with no cells added showed some DNA amplification. Additionally, it was hypothesized that cellular RNA was captured onto the beads as well, causing the second nonspecific band for samples that had cells. The unpredictability of these results led to an abandonment of this method in favour of a HotSHOT lysis and cellulose filter paper extraction, which produced much more repeatable results.

3.2 Using RCA to detect bacterial gDNA isolated on cellulose paper

Once the modified HotSHOT lysis and cellulose filter paper extraction procedure had been proven to be effective for detection of bacterial contamination in food and drink samples through PCR, a good test for applications to low-resource settings would be to apply this procedure to

RCA. This would require connecting a DNA circle and RCA primer to the genome, which was achieved using a linker sequence that was complementary to the genome on one end, and complementary to the DNA circle on the other end. Figure 3.2 shows a diagram of the system used for linkage of the circle followed by RCA and then detection.

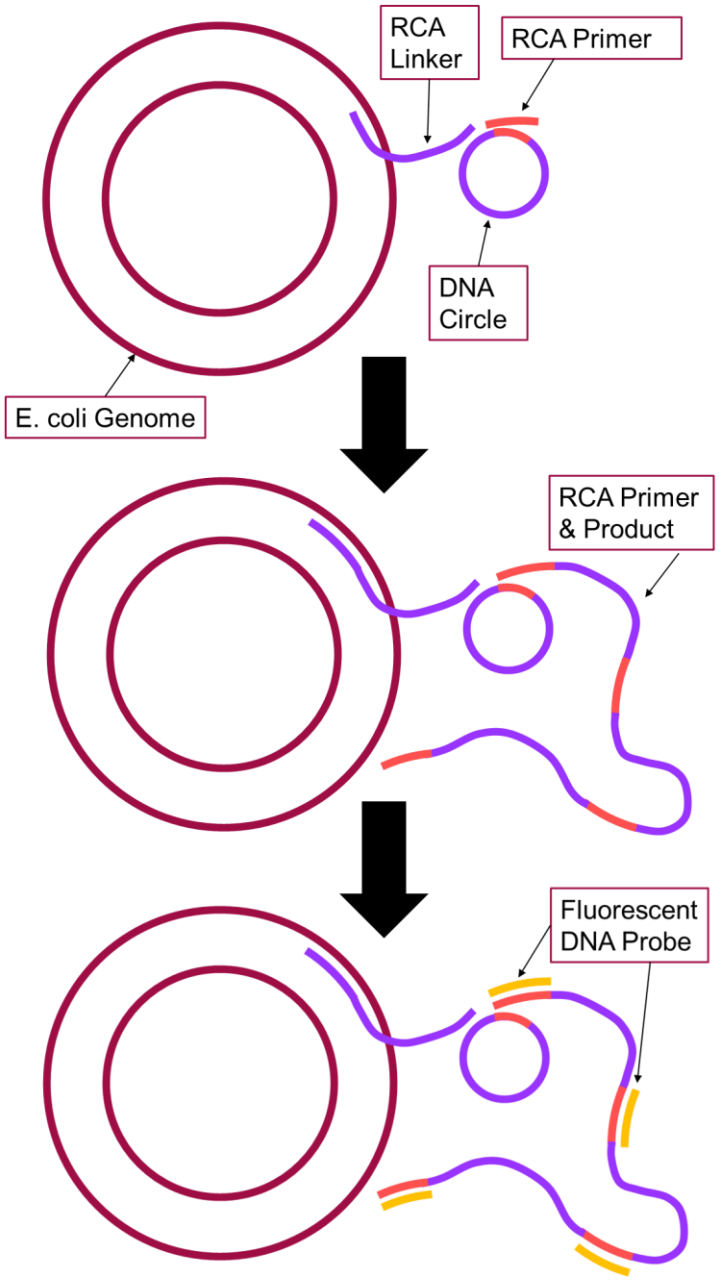


Figure 3.2 Schematic representation of the linkage of a DNA circle and RCA primer to bacterial gDNA during the HotSHOT reaction, followed by an RCA reaction and then fluorescent probe detection. This image is not to scale.

This reaction procedure was conducted by adding 1µL of a 1µM solution of each of RCA linker, circle, and primer (3µL total) to a tube, after which a HotSHOT lysis reaction was performed, as in Section 2.2.4. This was followed by a cellulose filter paper gDNA extraction as in Section 2.2.2. The nucleotide sequences used for these experiments are shown in Table 3.4 below. All of the oligonucleotides were ordered from Integrated DNA Technologies®.

Table 3.4 Sequences of oligonucleotides used to run an RCA reaction in association with captured *E. coli* gDNA.

Oligonucleotide	Sequence (5'-3')
Circle Linker	CAT CAT GCA AGC GGC CTC TGT TTT TTT TTT TTT TTG GCG AAG ACA GGT GCT TAG TC
Circle Template	TGT CTT CGC CTT GTT TCC TTT CCT TGA AAC TTC TTC CTT TCT TTC TTT CGA CTA AGC ACC
RCA Primer	GGC GAA GAC AGG TGC TTA GTC
RCA Probe	/56 FAM/TTC TTT CCT TGA AAC TTC TTC CT

At this point, it was theorized that the genome with the RCA circle and primer attached had been captured onto the paper microzones. This procedure was inspired by a study from McMaster that showed that RCA was possible on a nitrocellulose membrane, where the circular DNA template had been printed onto the paper itself[6]. Their protocol involved centrifugation for preparation of DNA for printing, followed by blocking the membrane surface with BSA after printing, and the necessity of these steps was examined since centrifugation, DNA printing, and creating and storing protein buffers is not conducive to low-resource laboratories. Thus, if it was possible to successfully capture and bind the genome with the RCA circle and primer attached, printing of nucleotides would not be required.

The experiment conducted to test this involved one control tube and three sample tubes in each test set, where the control had no linker, circle or RCA primer, and all three samples contained 1µL of a 1µM solution of these reagents. The first reaction set contained no cells at all. This was

to prove that the RCA product would not be attached to the paper microzones if no cells were present. The second reaction set contained 10^5 *E. coli* cells, and was used to prove that following the HotSHOT lysis and cellulose paper extraction, RCA would only occur if the linker, circle and RCA primer had been added during the initial lysis step of the HotSHOT reaction. The third and fourth reaction sets corresponded with repeats of the first and second sets, with an added step to immerse the paper microzones for 20 minutes in a 1X PBS buffer containing 10% w/v BSA, followed by drying the microzones at room temperature. RCA was then conducted simultaneously on all 4 reaction sets in a reaction volume of 15µL, with reagents added as specified in Table 3.5. Φ29 DNA Polymerase and 10mM dNTP Mix were purchased from Thermofisher Scientific. The RCA reaction was conducted for 40 minutes at room temperature, following which, 10µL of a 1µM solution of the fluorescent RCA probe was added and allowed to hybridize for 20 minutes at room temperature. After adding the RCA probe, all of the microzones were covered using aluminium foil to prevent photobleaching of the fluorescein marker by overhead lights. After the probe had been hybridized, the microzones were washed by repeated immersion in an unmodified 1X PBS buffer, to remove any unattached RCA product and probe, and the dried. Finally, all of the microzones were imaged using a BioRad™ ChemiDoc XRS+ fluorescence imager, using the setting for "Fluorescein Blots". Table 3.6 shows a compilation of the images generated by each reaction set from this experiment.

Table 3.5 Quantities of reagents added to each RCA

Reagent	Volume Added	Final Concentration
dNTP Mix (10 mM)	1.0 µL	200µM
10X Φ29 DNA Polymerase Buffer	1.5 µL	1X
Φ29 DNA Polymerase (5U/µL)	0.5 µL	1 unit/ 50µL
ddH_2O	12.0 µL	N/A

Table 3.6 Fluorescence image of RCA tests performed on microzones. All triplicate test samples contain RCA linker, circle and primer, whereas all controls do not. Reaction Sets 1 and 3 have no cells added, whereas Reaction Sets 2 and 4 have 10^5 *E. coli* cells added, including their respective controls. Reaction Sets 3 and 4 underwent blocking with a BSA solution before RCA.

Reaction Set	Control	Test Samples
1		
2		
3		
4		

These results demonstrate that the BSA blocking step is not necessary for this procedure, and can even cause false positives, as seen by a bright fluorescence in the control of Reaction Set 4, which contained 10^5 *E. coli* cells, but no RCA linker, circle or primer. Additionally, there is an increase of fluorescence intensity when comparing the reaction sets that were unblocked with their corresponding blocked reaction set. It was visually examined that blocking with BSA caused the formation of a thin crystalline layer on the surface of the microzone as well as the wax printed outline. Thus, it is hypothesized that there is an interaction between BSA and the RCA probe, which potentially caused the excess probe to be entrapped by BSA crystals so that it cannot be effectively washed out.

However, it is clear from Reaction Sets 1 and 2 that an unblocked cellulose paper microzone can be used to effectively isolate the gDNA bound to a linker, circle and RCA primer, and that this structure can be effectively used to generate an RCA product that will not be washed out and can be easily detected for 10^5 *E. coli* cells. Further tests should be conducted to determine a LoD for this experimental procedure which offers great potential for equipment-free detection of bacteria in low-resource settings.

Chapter 4: Conclusions & Future Recommendations

4.1 Summary of Work

A cellulose filter paper-based extraction step was coupled with a modified version of the HotSHOT lysis reaction as a rapid, low-cost method to effectively isolate bacterial gDNA from complex solid and liquid samples. This was then integrated into bacterial detection via nucleic acid amplification by simple PCR, and it was shown to produce a better signal than adding the cells themselves directly to PCR. Furthermore, the effectiveness of this paper-based pre-treatment step allowed for detection of less than 1000 cells from complex samples that would have otherwise caused complete inhibition of PCR. This simple sample treatment can be conducted in under 30 minutes and shows promise for speeding up nucleic acid testing for disease detection.

First, unmodified Whatman Grade 1 filter paper was used in conjunction with a wax printer to create a 96-well plate pattern, with well diameters of 4mm. Each well was cut into an 8mm square microzone with a wax boundary and unmodified center, and then placed onto a 5cm square of plain filter paper. This setup served as the extraction device. Lysis was conducted by incubation in a basic solution for 10 minutes followed by a neutralization, both at room temperature. Lysate was then flowed through the extraction device, followed by PBS buffer to act as a washing step. Each microzone was then suspended in water and vortexed, following which the water was used in a PCR targeting the specific bacterial strain of interest. PCR products were then analyzed through agarose gel electrophoresis. Soil, milk, lettuce and chicken breast were used as complex samples to test the applicability of this method at various stages of the food production chain. A standardized cell suspension was used to spike samples, and cell dilutions were used to calculate the LoD. The estimated limits of detection using simple PCR varied by sample type, but all were similar to that of the BAX® System Standard PCR Assays, which are approved by the Canadian

government, without the need for a lengthy bacterial enrichment step. This method was determined to be semi-quantitative in that it will detect above a threshold concentration of bacteria but will not provide a repeatable accurate quantification for the cell count in a sample, however, this is a common limitation with simple PCR assays.

It was also shown that the paper-based extraction procedure could be used in conjunction with RCA to detect 10^5 cells of *E. coli* in solution. However, this was not tested with complex samples, just a suspension of cells in PBS. A bead-based gDNA extraction was also attempted, however the results generated by this method in conjunction with PCR were not repeatable, so it was not explored further.

4.2 Direction for Future Work

The primary goal of this thesis was achieved, as this cellulose paper-based gDNA extraction has been demonstrated to be a quick, low-cost method for sample preparation that can be seamlessly integrated into nucleic acid testing for bacterial detection. However, there is still much work to be done to explore the many applications of this system.

Firstly, it would be useful to validate these findings with real world samples that have innate bacterial contamination and not spiked with bacteria in a laboratory setting. If a sample is deemed as contaminated by another detection method, this method could then be applied to an aliquot of that sample, which would also allow for a comparison to other currently used methods of detection.

It would also be worthwhile to expand this method to detect other important foodborne bacteria, or even food that had been fungally or virally contaminated. If these future advances succeed, this method can then be applied to a biosensing platform as a multiplex PCR cascade to semi-quantitatively test whether any of the relevant bacterial, fungal or viral contaminant levels

for a sample have been exceeded all in one test, rather than performing a separate test for each contaminant.

Next, it would be worthwhile to improve the quantifiability of PCR detection following a cellulose paper gDNA extraction. This could be achieved by using qPCR instead of simple PCR to be able to accurately determine the cell count in a sample based on the number of copies of gDNA. This would also allow for testing the efficiency of paper-based extraction, to see if there is any loss of genomic material in this step, and if so, what percentage of genomic copies get extracted. If both qPCR and mPCR work well, this system could show promise for a real-time detection method to determine an accurate count within a sample of different relevant foodborne bacteria, fungi and viruses. It would also be useful to test qPCR after bacterial enrichment followed by the simple extraction step, to determine the true lower limit of quantifiability of this system.

Since the vast majority of the global foodborne disease burden falls on the developing world, it is important to design rapid tests that are cost-effective and simple to perform with basic reagents for optimal detection in low-resource settings. As such, further exploration of our paper-based extraction method in conjunction with RCA should be conducted, especially the determination of an LoD, to see how it compares to other rapid bacterial detection methods. While there are still steps to be taken to develop a fully-integrated analytical procedure with RCA detection, the work done here has shown promise that such a system would be feasible, and demonstrated that the rapid procedure of a modified HotSHOT lysis coupled with a cellulose paper extraction can successfully extract bacterial gDNA in a manner that allows for specific amplification and detection.

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Appendix A: Supplementary Figures & Information

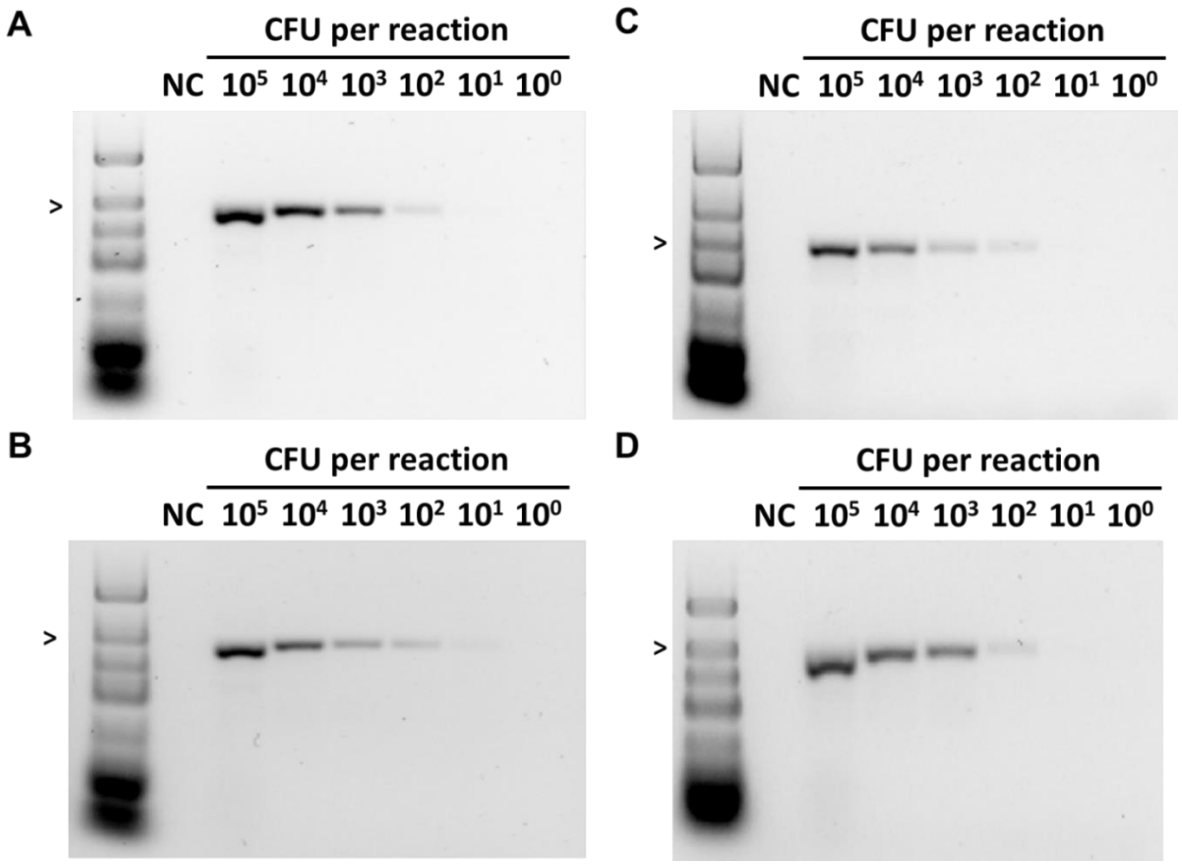

Figure A1.1 Replicates of the limit of detection PCR test for *E. coli* cell suspensions added directly to PCR.

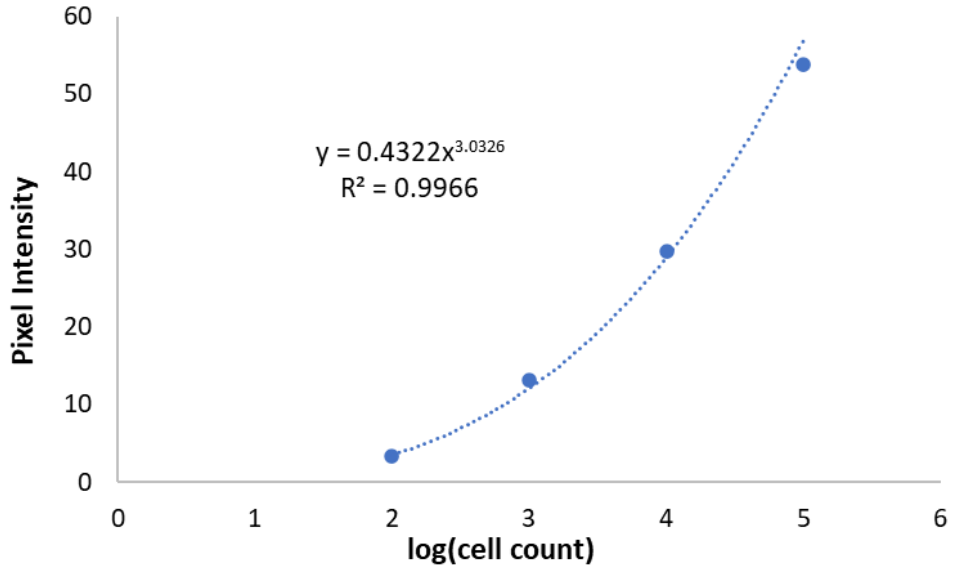

Figure A1.2 Calibration curve generated from the limit of detection PCR test for *E. coli* cell suspensions added directly to PCR.

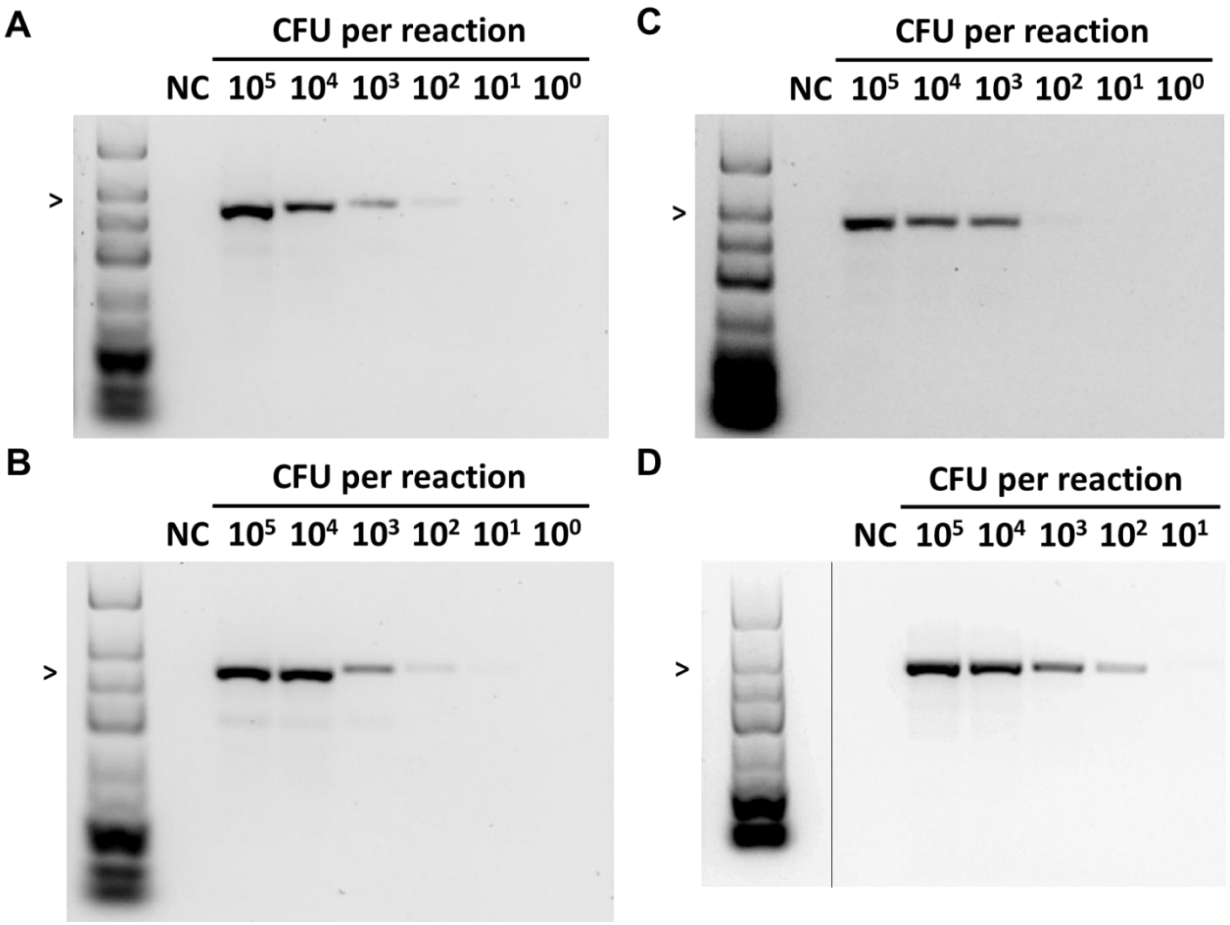

Figure A1.3 Replicates of the limit of detection PCR test for *E. coli* cell suspensions that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

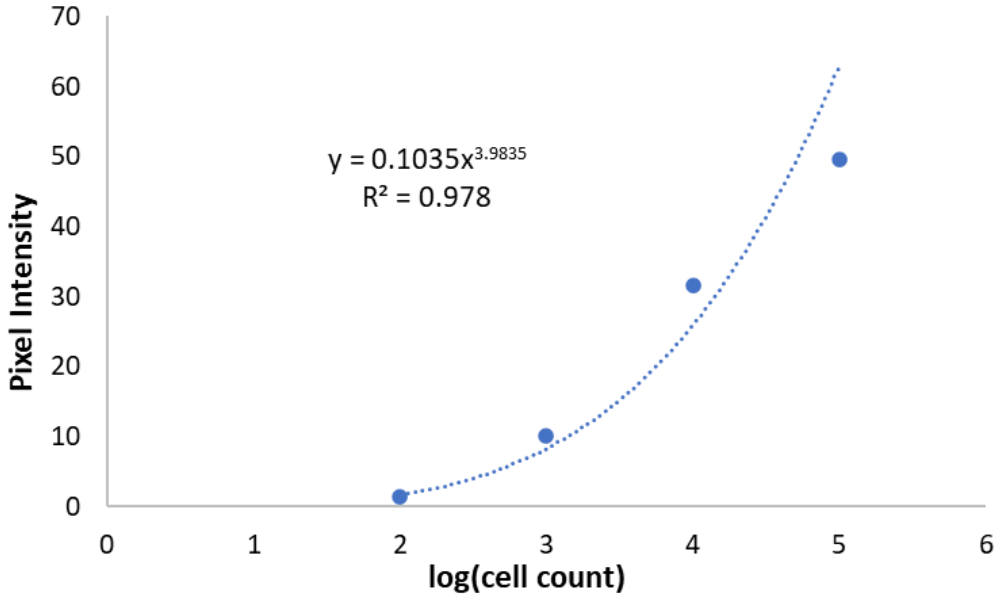

Figure A1.4 Calibration curve generated from the limit of detection PCR test for *E. coli* cell suspensions that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

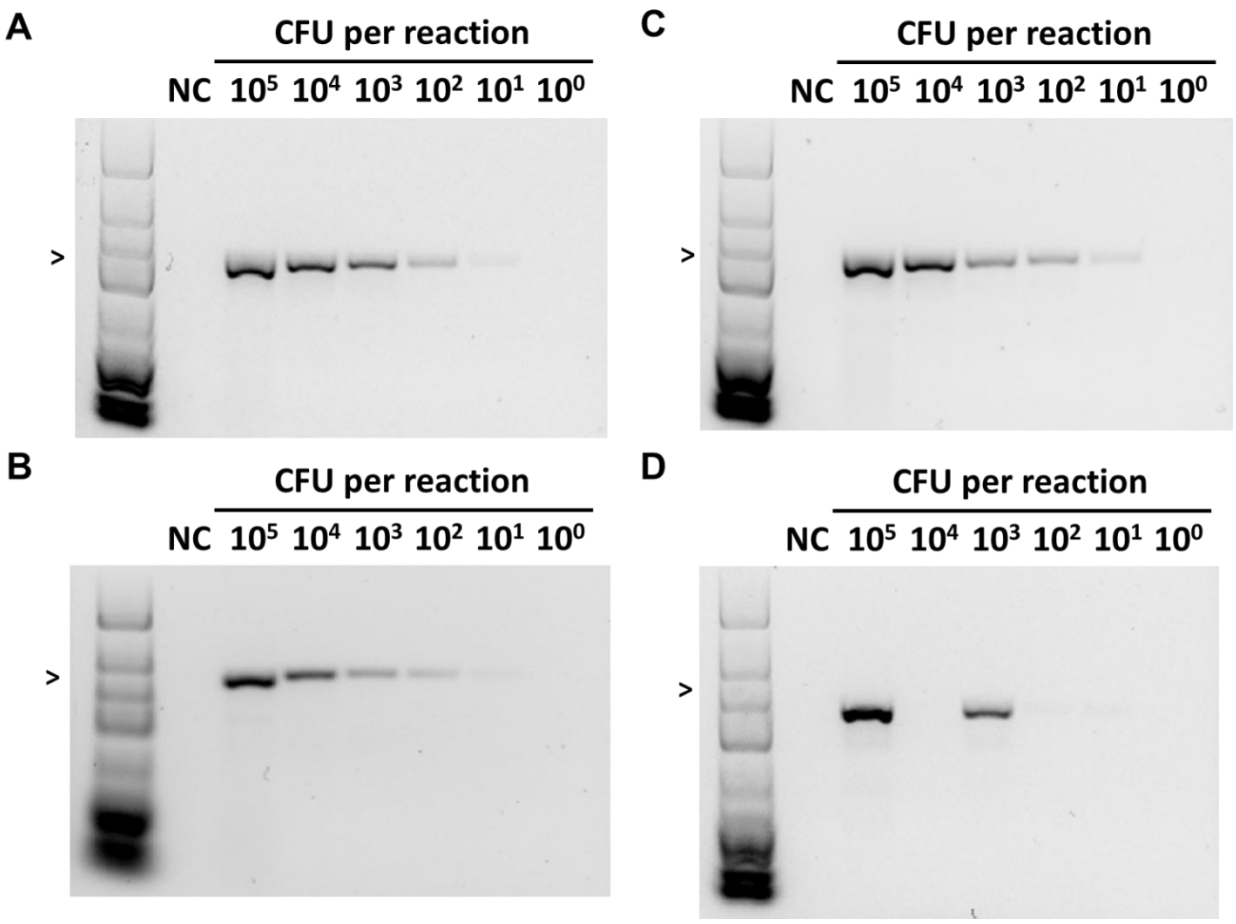

Figure A1.5 Replicates of the limit of detection PCR test for *S.* Newport cell suspensions added directly to PCR.

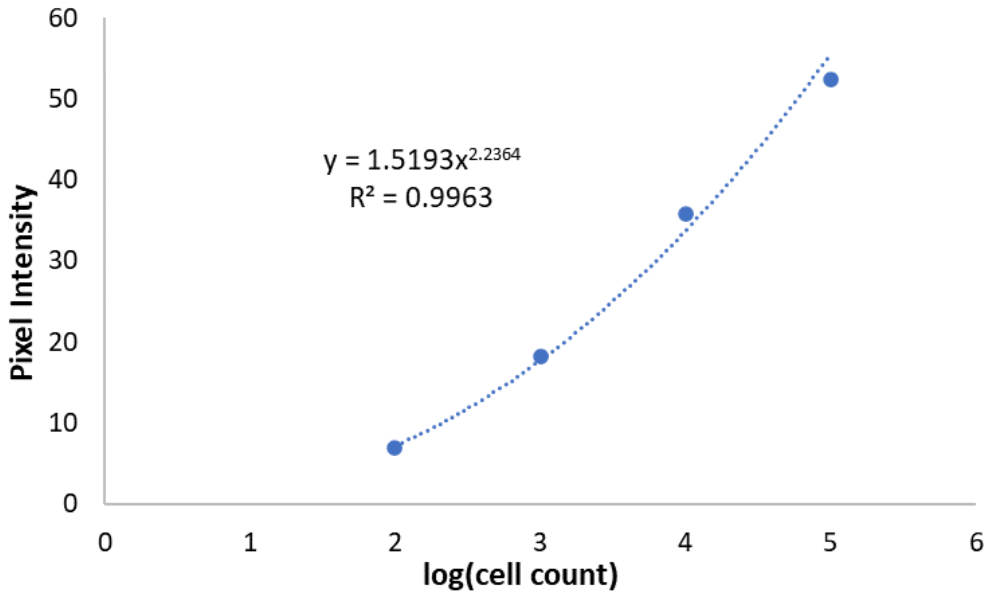

Figure A1.6 Calibration curve generated from the limit of detection PCR test for *S.* Newport cell suspensions added directly to PCR.

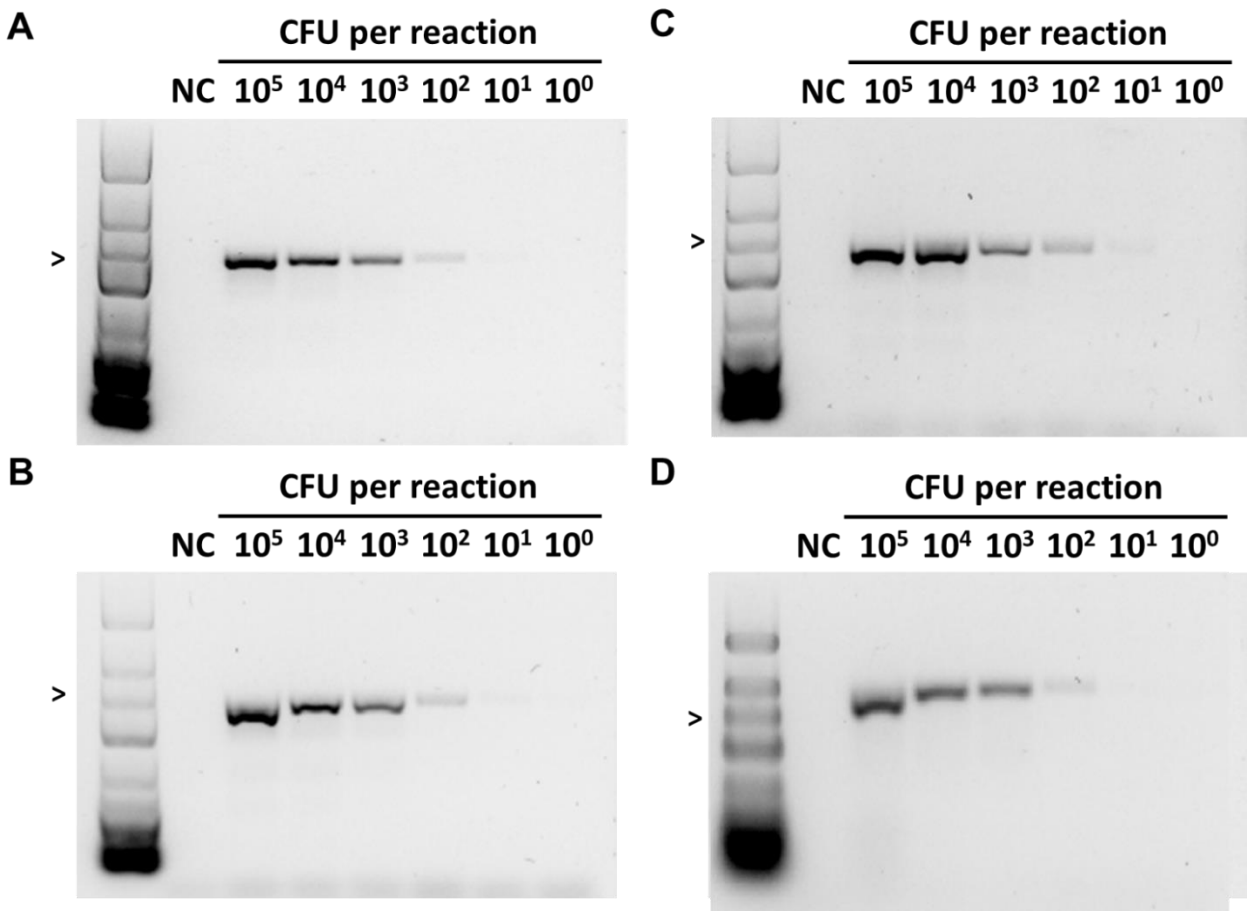

Figure A1.7 Replicates of the limit of detection PCR test for *S.* Newport cell suspensions that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

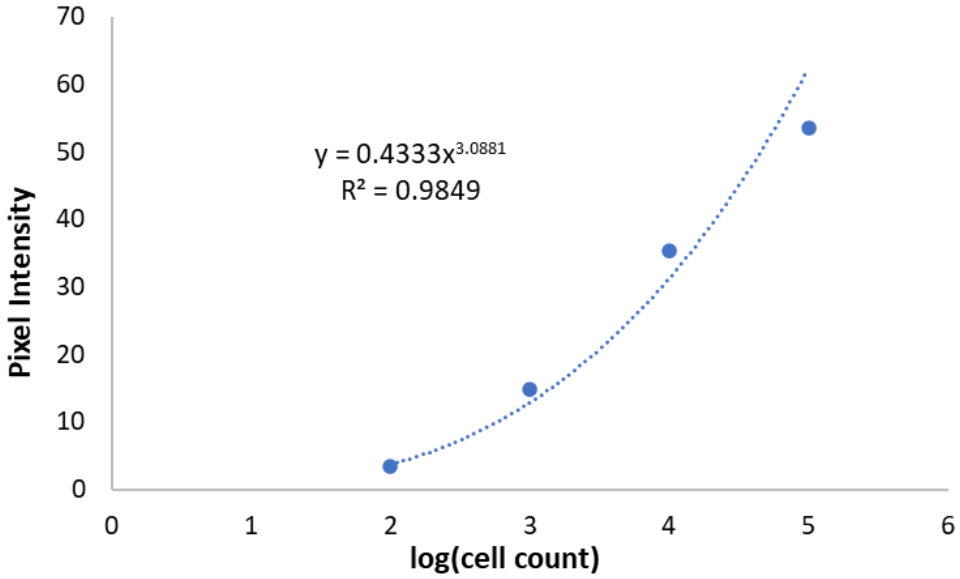

Figure A1.8 Calibration curve generated from the limit of detection PCR test for *S.* Newport cell suspensions that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

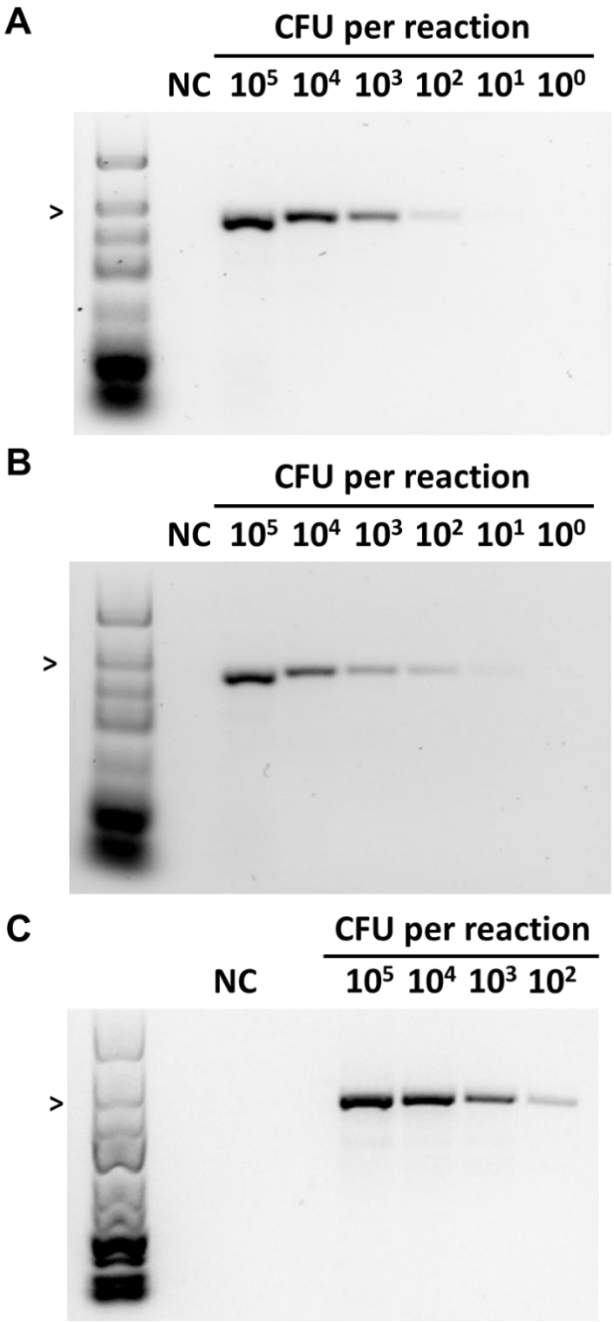

Figure A1.9 Replicates of the limit of detection PCR test for 20% w/v soil slurry samples spiked with *E. coli* cells that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

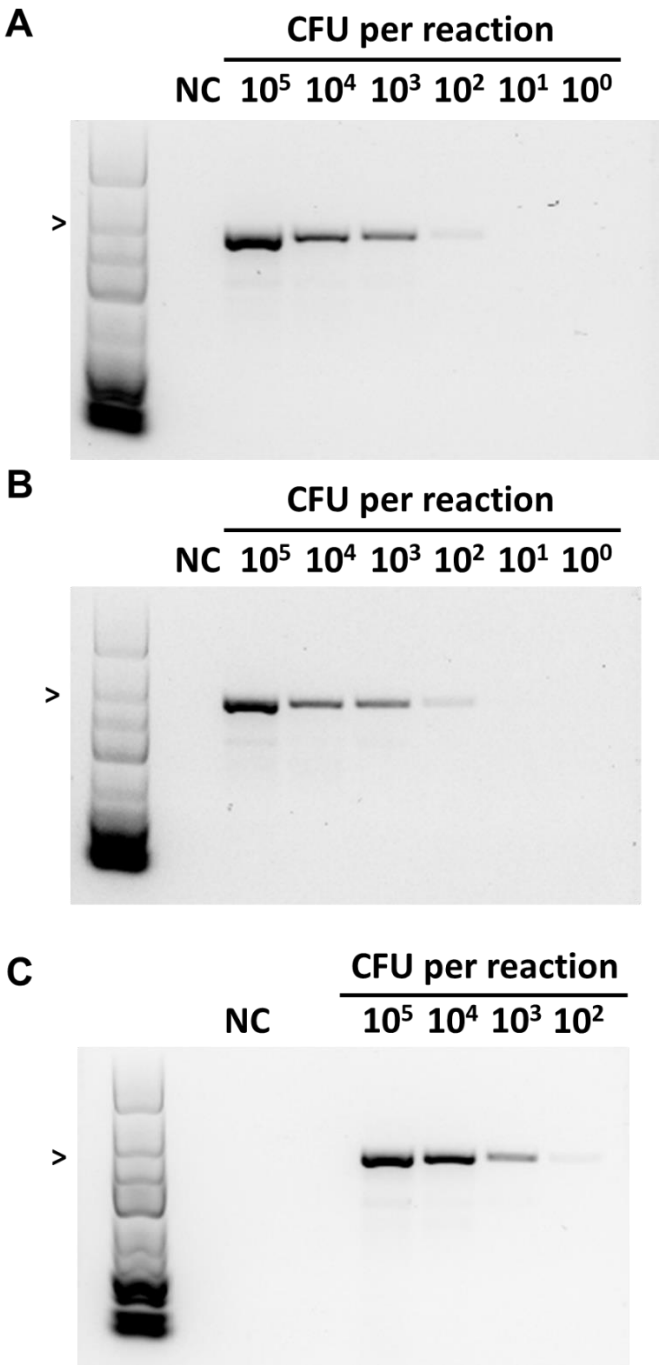

Figure A1.10 Replicates of the limit of detection PCR test for skim milk samples spiked with *E. coli* cells that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

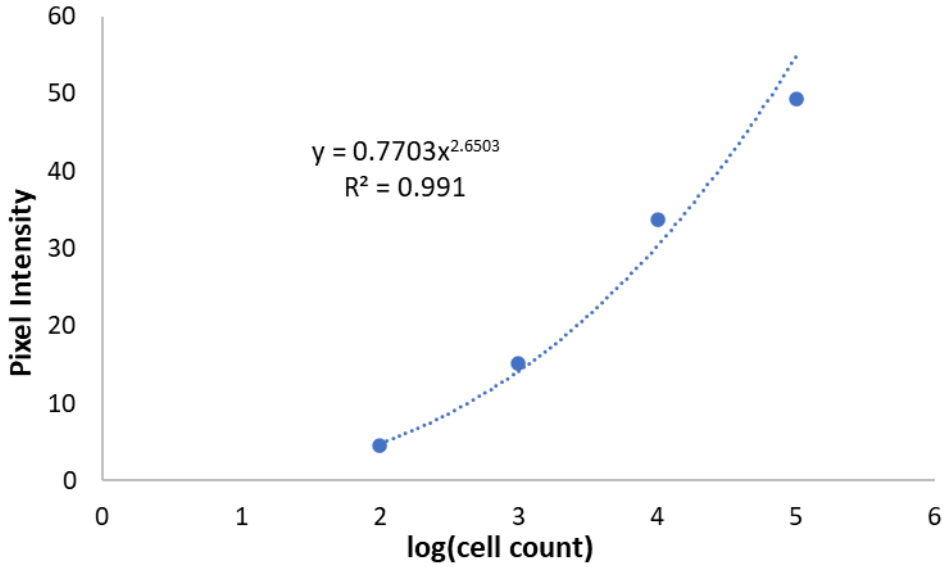

Figure A1.11 Calibration curve generated from the limit of detection PCR test for 20% w/v soil slurry samples spiked with *E. coli* cells that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

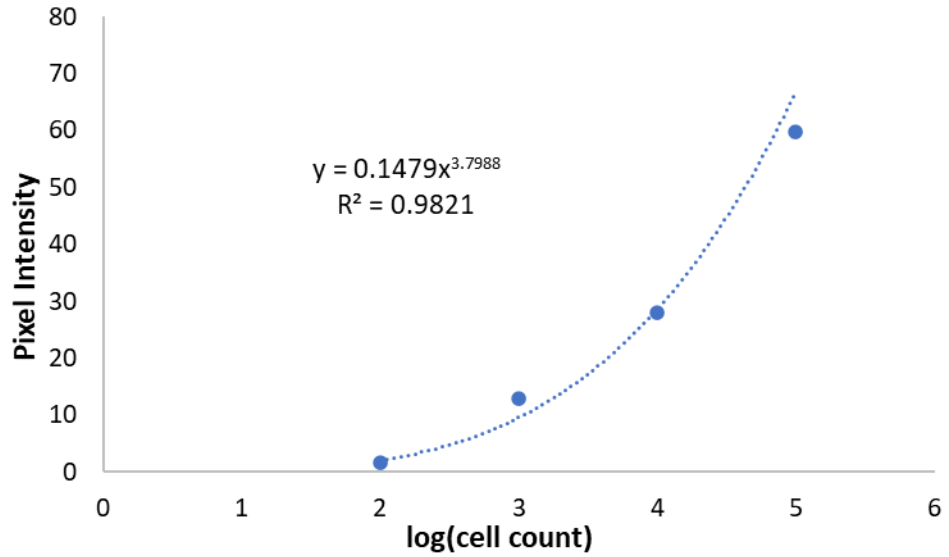

Figure A1.12 Calibration curve generated from the limit of detection PCR test for skim milk samples spiked with *E. coli* cells that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

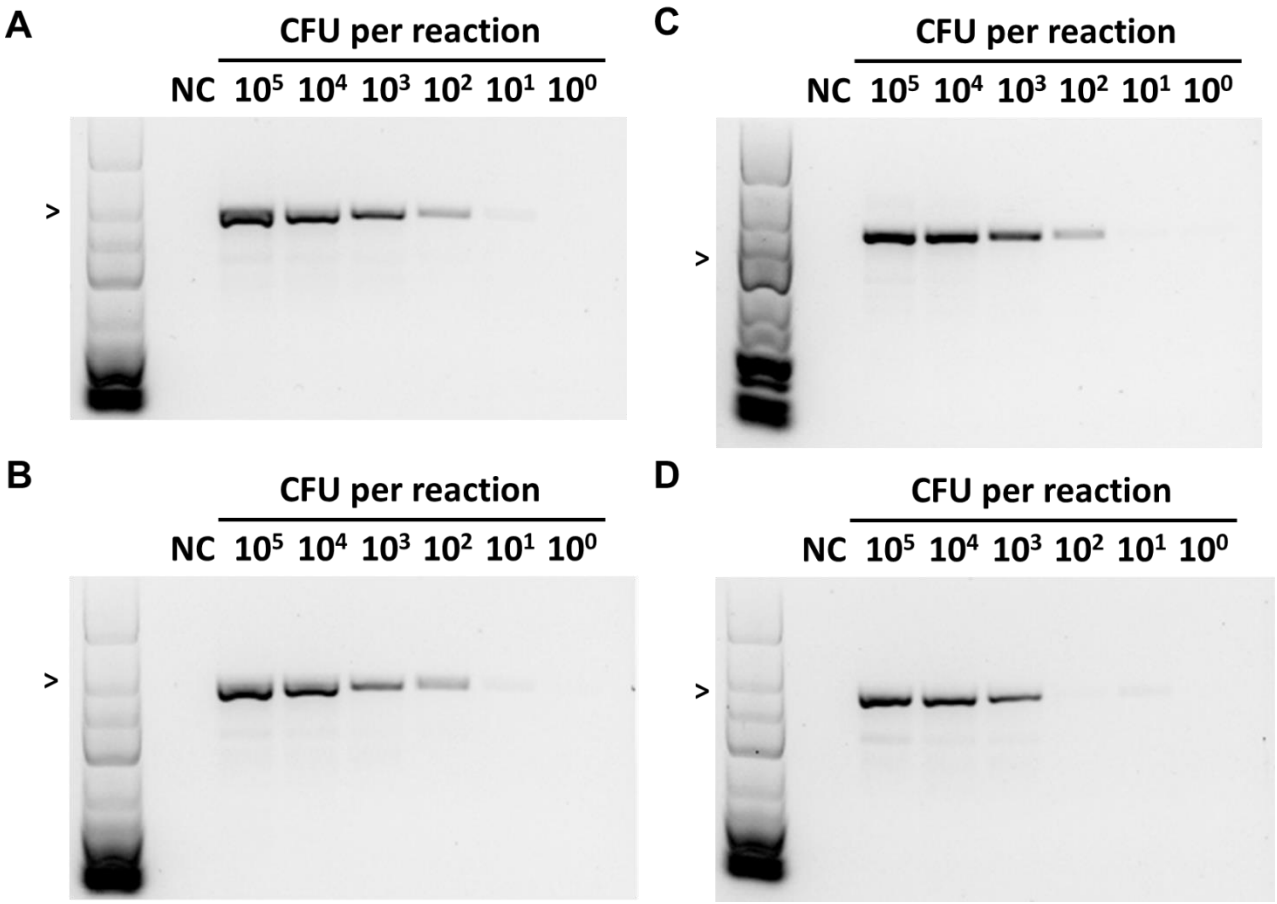

Figure A1.13 Replicates of the limit of detection PCR test for lettuce samples spiked with *E. coli* cells that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

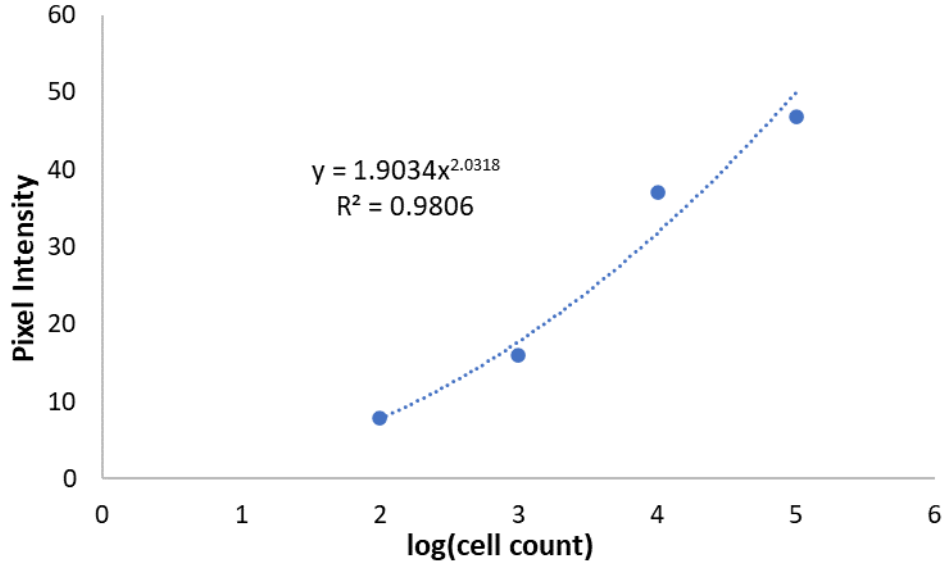

Figure A1.14 Calibration curve generated from the limit of detection PCR test for lettuce samples spiked with *E. coli* cells that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

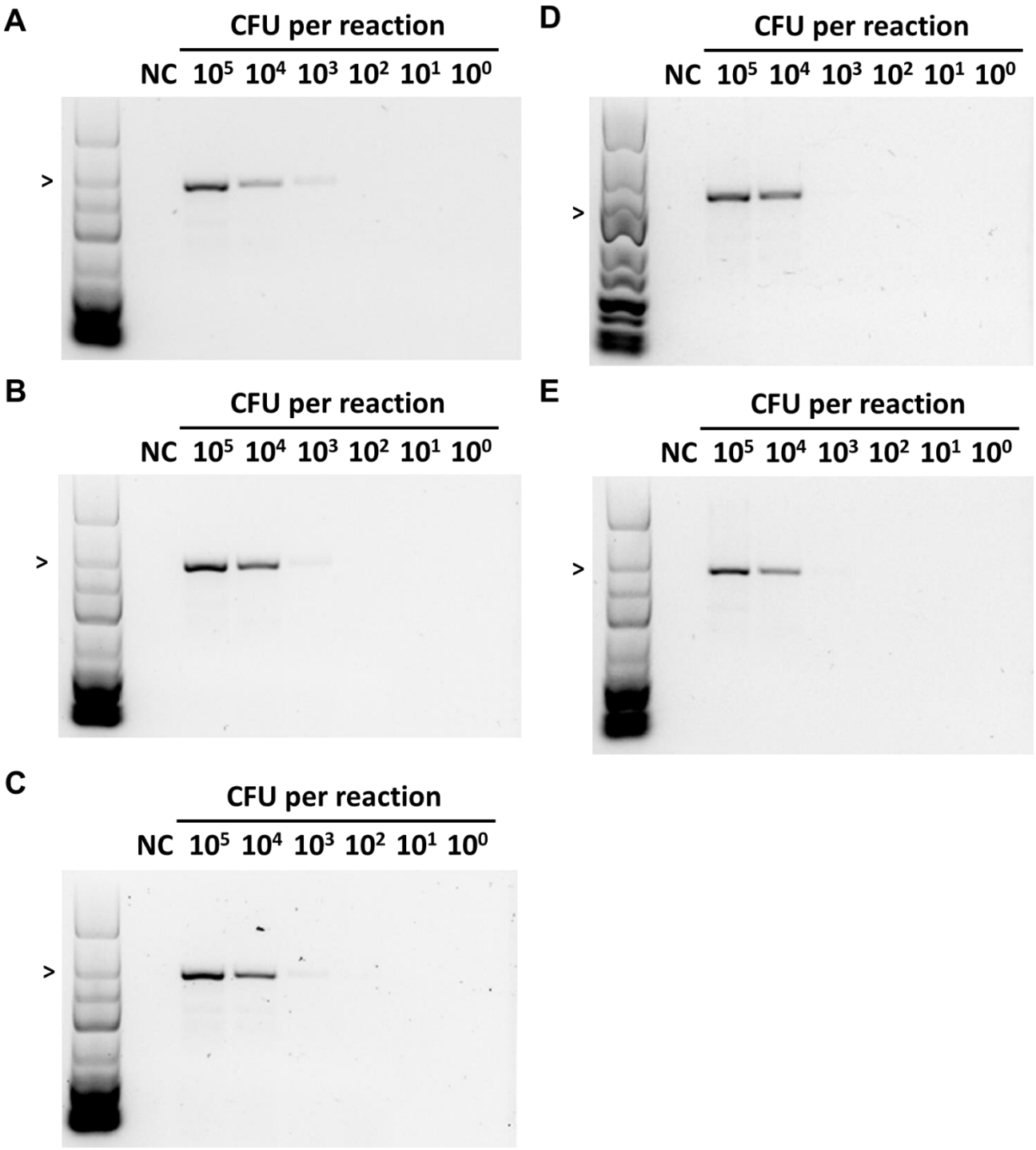

Figure A1.15 Replicates of the limit of detection PCR test for raw chicken samples spiked with *E. coli* cells that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

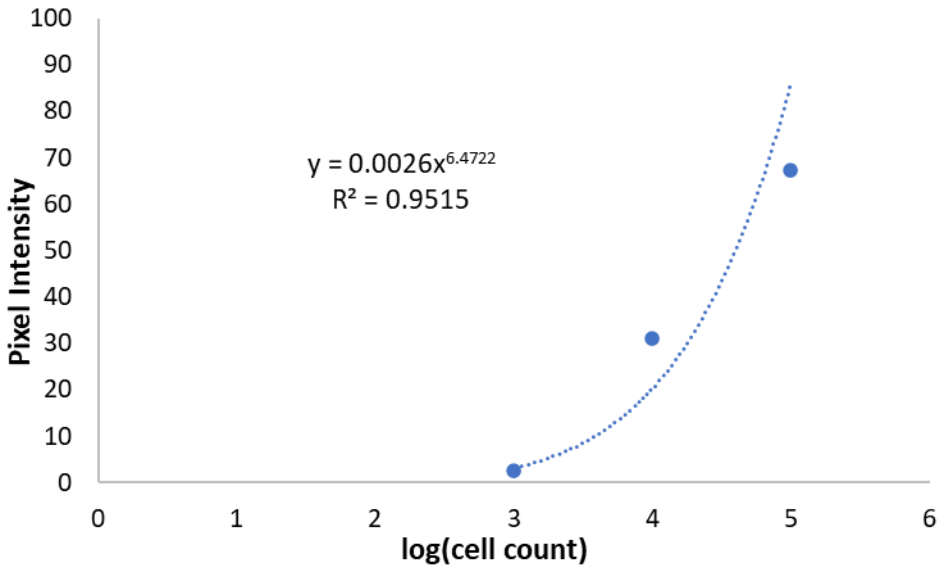

Figure A1.16 Calibration curve generated from the limit of detection PCR test for raw chicken samples spiked with *E. coli* cells that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

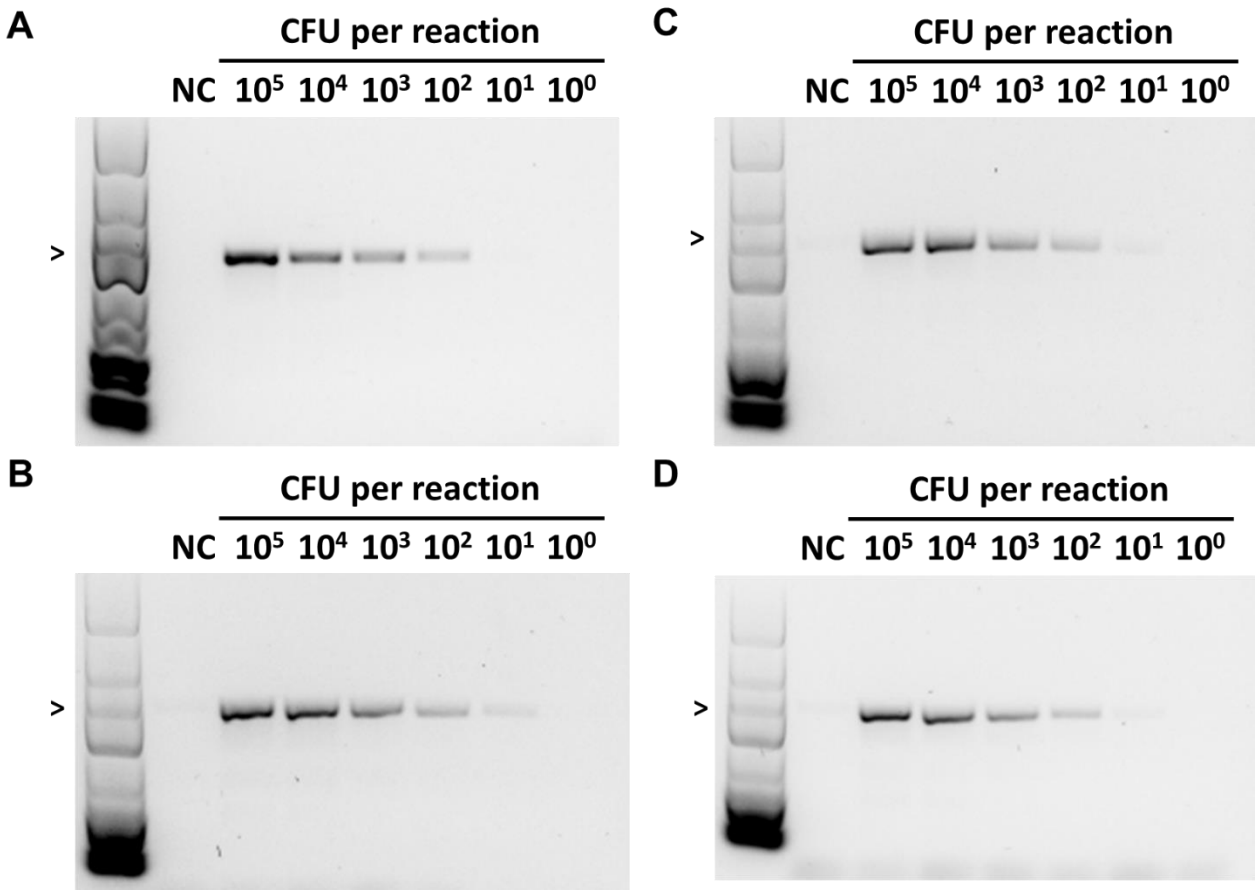

Figure A1.17 Replicates of the limit of detection PCR test for lettuce samples spiked with *S.* Newport cells that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

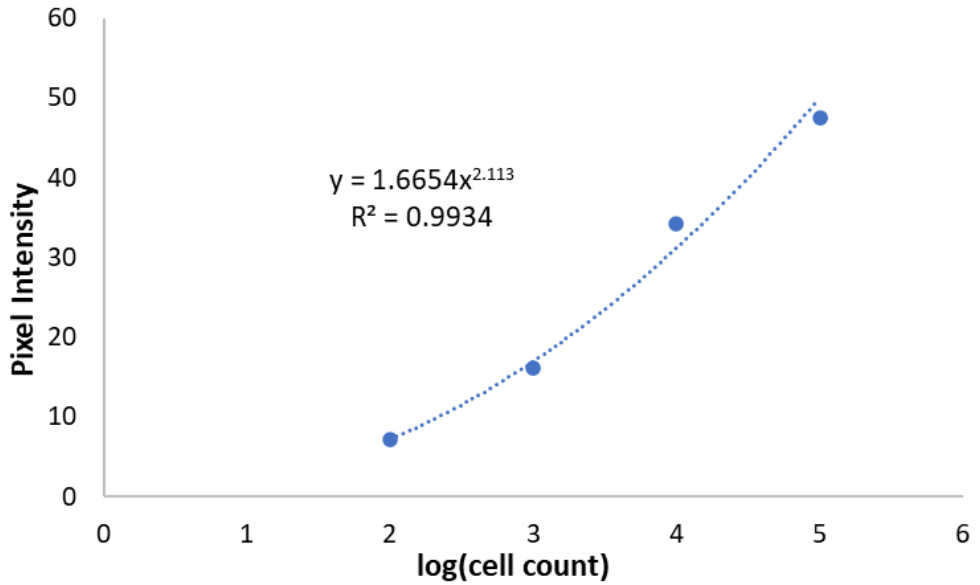

Figure A1.18 Calibration curve generated from the limit of detection PCR test for lettuce samples spiked with *S.* Newport cells that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

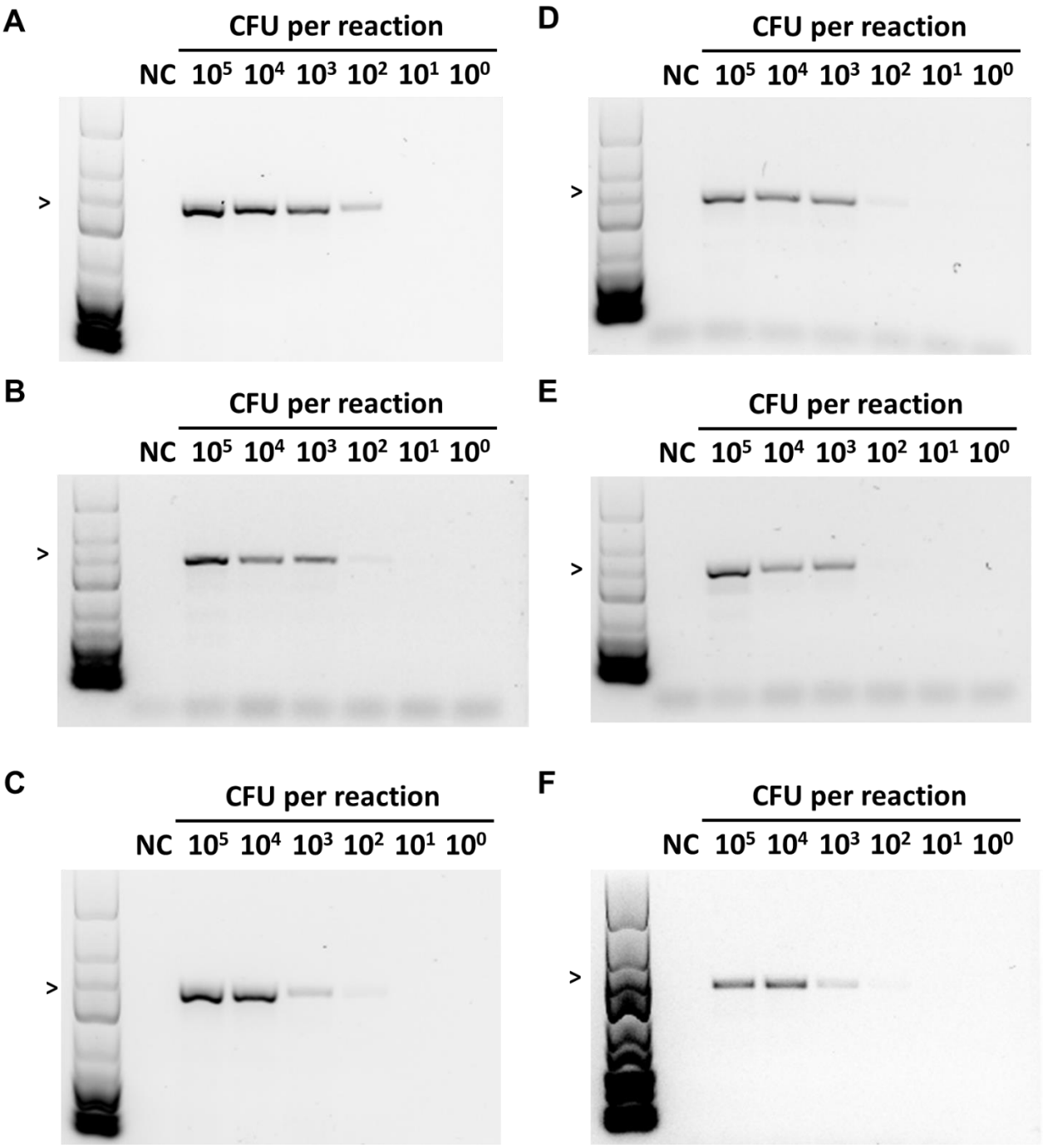

Figure A1.19 Replicates of the limit of detection PCR test for raw chicken samples spiked with *S.* Newport cells that underwent the modified HotSHOT lysis and paper filtration prior to PCR.

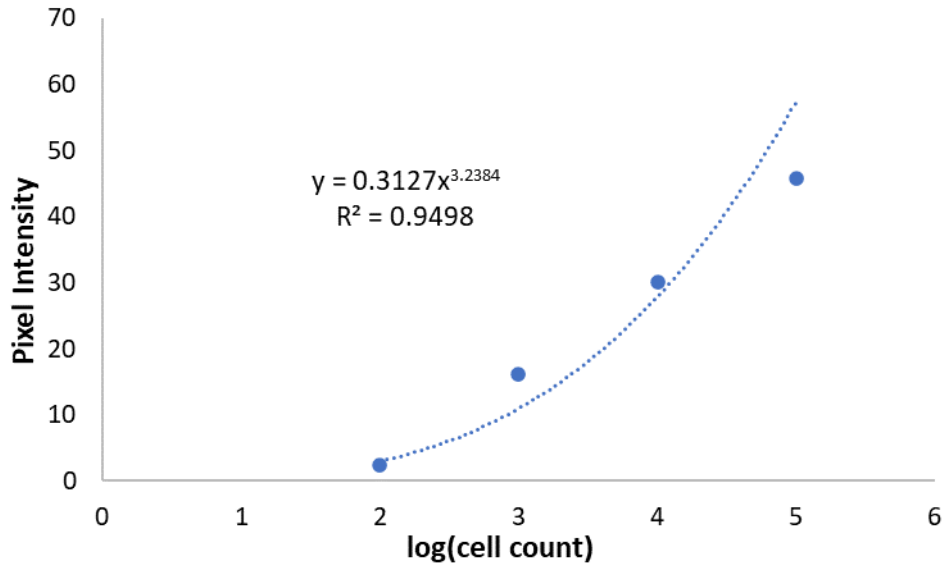

Figure A1.20 Calibration curve generated from the limit of detection PCR test for raw chicken samples spiked with *S.* Newport cells that underwent the modified HotSHOT lysis and paper filtration prior to PCR.